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EDITORIAL

Importance of gastrin in the pathogenesis and treatment of gastric tumors

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Abstract

In addition to regulating acid secretion, the gastric antral hormone gastrin regulates several important cellular processes in the gastric epithelium including proliferation, apoptosis, migration, invasion, tissue remodelling and angiogenesis. Elevated serum concentrations of this hormone are caused by many conditions, particularly hypochlorhydria (as a result of autoimmune or *Helicobacter pylori* (*H pylori*)-induced chronic atrophic gastritis or acid suppressing drugs) and gastrin producing tumors (gastrinomas). There is now accumulating evidence that altered local and plasma concentrations of gastrin may play a role during the development of various gastric tumors. In the absence of *H pylori* infection, marked hypergastrinemia frequently results in the development of gastric enterochromaffin cell-like neuroendocrine tumors and surgery to remove the cause of hypergastrinemia may lead to tumor resolution in this condition. In animal models such as transgenic INS-GAS mice, hypergastrinemia has also been shown to act as a cofactor with *Helicobacter* infection during gastric adenocarcinoma development. However, it is currently unclear as to what extent gastrin also modulates human gastric adenocarcinoma development. Therapeutic approaches targeting hypergastrinemia, such as immunization with G17DT, have been evaluated for the treatment of gastric adenocarcinoma, with some promising results. Although the mild hypergastrinemia associated with proton pump inhibitor drug use has been shown to cause ECL-cell hyperplasia and to increase *H pylori*-induced gastric atrophy, there is currently no convincing evidence that this class of agents contributes towards the development of gastric neuroendocrine tumors or gastric adenocarcinomas in human subjects.

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Key words: *Helicobacter pylori*; Hypergastrinemia; Neuroendocrine; Gastric carcinoma; Proton pump inhibitor

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INTRODUCTION

Gastric epithelial malignancy represents a significant burden of disease. The commonest lesion is gastric adenocarcinoma, which is the fourth commonest malignancy worldwide, and because it is associated with a high mortality, this tumor remains the second commonest cause of cancer-related death globally. The distribution of gastric epithelial malignancies is not uniform between different populations, with increased prevalence being found in East Asia including Japan and China (where 42% of cases occur), as well as in Eastern Europe and South America[1]. This epidemiology raises significant questions as to the predisposing factors for gastric malignancy. Current data suggest that a number of different variables affect an individual's risk of gastric carcinogenesis, amongst which are environmental

factors such as infection with *Helicobacter pylori* (*H pylori*), smoking and diet, as well as host factors such as achlorhydria and specific cytokine polymorphisms. Another important host factor which may play a role during gastric carcinogenesis is the hormone gastrin.

As well as acting as a potential cofactor during gastric adenocarcinoma development, gastrin is also known to play a major role in the pathogenesis of other gastric tumor types, particularly neuroendocrine (carcinoid) tumors. There is therefore accumulating evidence that gastrin not only influences tumor development, but could also be a potential therapeutic target for various gastric neoplasias. These issues will be the main focus of this editorial.

GASTRIN BIOCHEMISTRY AND PHYSIOLOGY

Synthesis and processing

The presence of a hormone that stimulated gastric acid secretion in the pyloric mucosa was first demonstrated in 1906[2]. Gastrin was subsequently shown to be secreted from neuroendocrine G cells which are principally located in the antrum of the stomach. The gastrin gene is located on the long arm of chromosome 17 and encodes a 101 amino acid polypeptide, preprogastrin. This gene product is subjected to a series of post translational modifications which result in the synthesis of a number of biologically active peptides[3] (Figure 1). Immediately after translation, preprogastrin is cleaved to form progastrin, which is transported to the Golgi network, where it is packaged into secretory vesicles. Further post-translational modification occurs at this site, firstly to form gastrin-34-Gly, the C-terminal glycine extended form of gastrin-34. This peptide may then undergo further cleavage into gastrin-17-Gly or amidation to generate gastrin-34, which in turn may be cleaved to form gastrin-17. Progastrin, glycine extended gastrins and amidated gastrins are all biologically active and exert different functions within gastric and other mucosae. Progastrin and glycine extended gastrins act particularly on the colonic mucosa as mitogens[4,5], glycine extended and amidated gastrins have been shown to affect the differentiation of gastric oxyntic mucosa, whilst amidated gastrin promotes cell proliferation as well as acid secretion in the stomach. In the human stomach, the conditions for post-translational modification of gastrin are such that there is almost complete amidation of glycine extended forms of gastrin, hence the predominant form of secreted gastrin is gastrin-17[3].

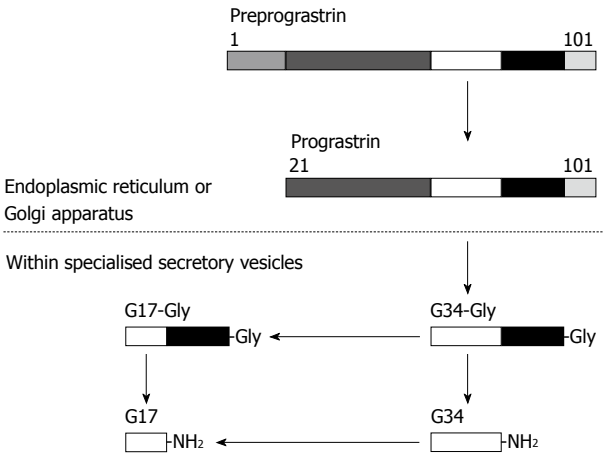

Figure 1 Biosynthesis of gastrin.

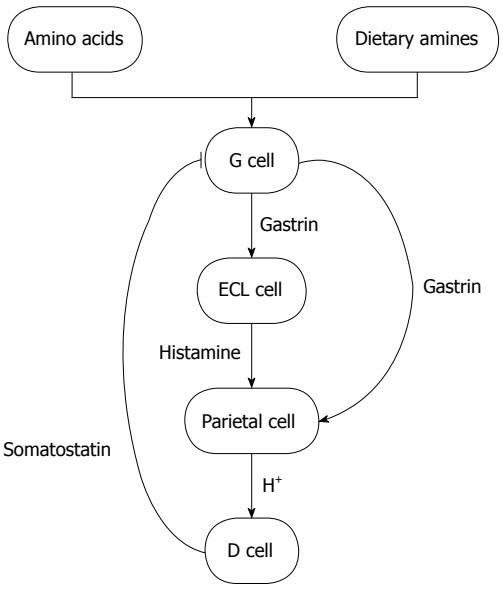

Figure 2 Mechanism by which gastrin regulates gastric acid secretion.

Cellular effects of gastrin

Acid secretion: Gastrin is secreted in response to a number of luminal stimuli, including the presence of amino acids and dietary amines (reviewed in[3]). Calcium receptors on the surface of the G-cell also sense luminal calcium and modulate the gastrin secretory response[6], with increased calcium resulting in increased gastrin secretion. Following secretion into the gastric vasculature, gastrin binds to CCK-2 receptors, which are expressed on the surface of gastric enterochromaffin-like (ECL) cells and parietal cells[7] (Figure 2). The predominant mode of secretagogue action is *via* ECL cell secretion of histamine and this pathway effectively amplifies the prosecretory signal. Continued gastrin secretion is negatively regulated by the secretion of somatostatin *via* D-cells, which are located in the oxyntic mucosa. In physiological states, these mechanisms maintain appropriate gastric pH.

Proliferation: Studies in dogs in 1972 provided the first evidence of mucosal proliferation in response to gastrin[8], however these studies were triggered by earlier clinical observations of increased gastric mucosal proliferation in patients with Zollinger-Ellison syndrome (ZES). Subsequently, increased fundic mucosal proliferation was demonstrated in rodent models including *Mastomys* following the administration of an H2 receptor antagonist which rendered them hypergastrinemic. These animals demonstrated gastric

gland elongation and increased numbers of cells which stained for Ki67, a marker of proliferation[9]. In normal humans, the infusion of gastrin at supraphysiological levels has also been shown to result in increased gastric cell proliferation, as demonstrated by ^{3}H-thymidine labelling studies[10].

Endocrine cell proliferation in the stomachs of patients with ZES was first reported in 1974[11]. Further evidence that hypergastrinemia provides a proliferative drive to ECL cells emerged from studies in which rats were rendered hypergastrinemic by treatment with either proton pump inhibitor (PPI) or H2 receptor antagonist drugs. ECL cell hyperplasia was not observed in control untreated animals or in rats that had been antrectomized prior to treatment with high-dose PPI[12]. These observations in rats have to some extent been corroborated in other species, as ECL cell hyperplasia has now also been demonstrated following PPI treatment of chickens, hamsters and guinea pigs[13,14]. ECL cell hyperplasia of this magnitude does not however appear to occur in mice or humans treated with acid suppressant drugs.

To investigate the molecular mechanisms by which gastrin promotes proliferation, a number of gastric cancer cell lines that express the CCK-2 receptor have been employed. It has recently been shown that the proliferation of MKN-45 cells, which are derived from a poorly differentiated gastric carcinoma and which have been reported to express the CCK-2 receptor, decreased when treated with the CCK-2 receptor antagonist AG-041R[15]. Several cell lines which have independently been stably transfected with the CCK-2 receptor (but using different expression vectors) have been generated from AGS gastric cancer cells (which do not constitutively express the CCK-2 receptor). The AGS-B cell line (transfected with human full-length CCK-2 receptor using the pcDNA I vector and neomycin selection) was found to proliferate more rapidly in the presence of gastrin, a process that was associated with the upregulation of cyclin D1[16]. In contrast, the effects of gastrin on the proliferation of AGS-G$_R$ cells (transfected with the human full length CCK-2 receptor driven by the EF1α promoter under puromycin selection) were more complex. When cultured in the presence of gastrin-17, AGS-G$_R$ cells showed a reduced rate of proliferation, an effect that was abrogated by the addition of a CCK-2 receptor antagonist. However, when AGS-G$_R$ cells were co-cultured with AGS cells that had been transfected with a green fluorescent protein producing construct (AGS-GFP cells) in the presence of serum-free medium, gastrin exposure caused an increase in the proliferation of the AGS-GFP cells. This suggests that gastrin treatment of AGS-G$_R$ cells results in the secretion of growth factors that are capable of acting in a paracrine manner to stimulate the proliferation of AGS-GFP cells. Further analysis of the underlying mechanisms showed that epidermal growth factor ligands, particularly heparin binding epidermal growth factor (HB-EGF) were involved[17].

HB-EGF promotes cell cycling and is overexpressed by a number of cancer cell lines, including some gastric cancer cell lines and is of particular relevance to this article as it provides a potential molecular link between *H pylori* infection, gastrin and increased cell proliferation[18]. *H pylori* infection of gastric cancer cell lines has been shown to significantly increase HB-EGF levels and this effect is dependent upon the presence of the CCK-2 receptor. The most likely explanation is that *H pylori* induces cells to secrete gastrin, which in turn binds to and activates the CCK-2 receptor, resulting in HB-EGF secretion. This hypothesis is supported by evidence from animal models, as INS-GAS hypergastrinemic mice also overexpress HB-EGF in the premalignant lesions which develop following *Helicobacter felis* (*H felis*) infection[19]. This may partly explain the observed synergy between hypergastrinemia and *Helicobacter* infection during gastric carcinogenesis in this animal model.

Apoptosis: Apoptosis is a fundamental cellular process which is often dysregulated during the development of malignancy. There is accumulating evidence that gastrin modulates the apoptosis of both normal and transformed gastric epithelial cells and these mechanisms may contribute towards tumor development.

Several models of carcinogenesis are based upon the somatic mutation hypothesis proposed by Vogelstein in relation to colon cancer[20]. This hypothesis suggests that sequential defects are acquired by a tissue stem cell, eventually leading to the development of dysplastic and malignant phenotypes. This hypothesis suggests that an early failure in targeting mutated stem cells for apoptosis allows them to survive and generate mutated clones which progress to cancer. In the stomach however, an alternative mechanism of adenocarcinoma development has recently been proposed, as the gastric cancers which arise in *H felis* infected mice appear to develop in tissue that was originally derived from bone marrow stem cells. Houghton, Wang and colleagues studied C57BL/6 mice which had undergone bone marrow transplantation from GFP producing mouse strains or ROSA26 mice, thus allowing tissues that originated from the bone marrow rather than preexisting mucosal stem cells to be observed[21]. Following *H felis* infection, gastric cancers developed in these mice at a similar rate to other *H felis*-infected C57BL/6 mice, however, the tumors arose in glands which produced GFP, indicating that the tissue was initially derived from the bone marrow transplant. The authors propose that gastric stem cells undergo apoptosis as a result of *Helicobacter* infection, and that the stem cell niche is replaced by pluripotent stem cells which were originally located in the bone marrow. These cells may be more susceptible to malignant transformation than the previously incumbent gastric epithelial stem cells.

Gastrin signalling *via* the CCK-2 receptor appears to increase the susceptibility of normal gastric epithelial cells *in vivo* to undergo apoptosis. In *Mastomys* treated with H2 antagonist drugs, a two-fold increase in apoptosis was observed in hypergastrinemic animals relative to controls[9]. In addition, when

mice were subjected to 12 Gy gamma irradiation or *Helicobacter* infection, increased numbers of apoptotic cells were observed in the gastric corpus mucosa of hypergastrinemic animals[22,23]. Increased radiation-induced apoptosis was demonstrated both in transgenic INS-GAS mice and also in a model of drug-induced hypergastrinemia involving FVB/N mice treated with omeprazole. Treatment with a CCK-2 receptor antagonist abolished the observed increases in gastric epithelial apoptosis in both cases, suggesting that this response is both gastrin and CCK-2 receptor dependent. In gastric biopsy samples obtained from humans with both *H pylori* infection and hypergastrinemia, similar observations of increased apoptosis were also made[23]. These results suggest that hypergastrinemia may increase the susceptibility of gastric epithelial stem cells to undergo apoptosis, thus permitting the engraftment of bone marrow derived cells as suggested by Houghton and Wang[21].

Antiapoptotic effects of gastrin have however also been described, particularly in transformed cell types. For example, amidated gastrin inhibits the apoptosis of rat pancreatic acinar cancer cells (AR42J), by signalling through a CCK-2 receptor and an AKT-mediated mechanism[24,25]. Similarly, the human gastric cancer cell line MKN-45 has been shown to be more susceptible to apoptosis when treated with a CCK-2 receptor antagonist and this was associated with upregulation of Bax and downregulation of Bcl-2[15]. More recently, gastrin has also been shown to inhibit AGS-G$_R$ cell apoptosis in a manner dependent upon the expression of the anti-apoptotic protein mcl-1. Increased mcl-1 expression was also observed in the type 1 gastric neuroendocrine tumors of hypergastrinemic patients[26].

The effects of gastrin on gastric apoptosis therefore appear to depend upon the underlying physiological and pathological conditions. There are thus a number of important questions which still need to be answered, not only regarding the effects of gastrin on apoptosis in differing circumstances, but also regarding the role of apoptosis during the development of gastric carcinoma and other gastric malignancies.

Angiogenesis: Angiogenesis is an essential feature required for tumor survival. In a number of GI malignancies, cyclooxygenase (COX)-2, an important rate-limiting step in the prostaglandin synthesis pathway, has been implicated in enhancing angiogenesis. For example, in human studies, COX-2 expression has been associated with the development of a more dense microvasculature around gastric tumors[27]. Gastrin has been shown to enhance COX-2 secretion in AGS-E cells (which have been stably transfected with the full-length human CCK-2 receptor on a EF1α promoter, but by a different research group from those who produced AGS-G$_R$ cells) *via* an Akt-dependent mechanism[28]. Studies of patients with atrophic gastritis secondary to chronic *Helicobacter* infection have shown that, in addition to elevated levels of gastrin, these patients also have significantly higher levels of COX-2 mRNA compared to unaffected controls, a phenomenon that is reversed after *H pylori* eradication[29]. The mechanisms mediating increased COX-2 transcription in this setting have not been fully investigated to date, and it is not clear whether this effect is solely related to hypergastrinemia associated with *Helicobacter* infection and gastric atrophy or whether additional independent factors also influence COX-2 production.

Case control studies of patients taking aspirin and other COX inhibiting drugs have demonstrated a reduction in relative risk not only of gastric cancer, but also of colon cancer. Aspirin now has an established role in colon cancer prevention, however, its role in preventing diseases of the gastric epithelium is less convincing, due in part to the increased risk of GI bleeding associated with its use. Selective COX-2 inhibitors may have some potential as gastric cancer chemopreventive agents, however the serious cardiovascular side effects associated with the long-term use of the current generation of these drugs makes it unlikely that they will be adopted for this purpose.

In addition to the potential indirect role of gastrin upon angiogenesis *via* COX-2 expression, gastrin has also been suggested to have direct effects upon angiogenesis using an *in vitro* system. When human umbilical vein endothelial cells (HUVECS) were seeded onto fibroblast monolayers, they formed vascular structures in response to various angiogenic stimuli, including both amidated and glycine extended forms of gastrin. This response was associated with increased production of HB-EGF and with elevated levels of matrix metalloproteinase (MMP)2, MMP3 and MMP9[30].

Migration and invasion: Other fundamental cellular processes which are involved in promoting carcinogenesis and that are modulated by hypergastrinemia are tissue remodeling and invasion. Gastric cancer development involves extensive remodeling of the gastric mucosa during a hypergastrinemic premalignant phase (atrophic gastritis), in order to institute the conditions required for gastric carcinogenesis. Similar mechanisms are thought to be responsible for the local invasion and metastasis that occur in frankly malignant lesions. These processes are complex and are controlled by numerous different mechanisms, however, gastrin does appear to play a regulatory role. For example, gastrin has been shown to increase levels of MMP-9 *via* an MAPK AP-1 dependent pathway in human patients with gastric cancers and gastric neuroendocrine tumors, resulting in tissue remodelling and invasion[31]. Serum levels of MMP-7 have also been found to be elevated in patients who were hypergastrinemic as a result of either MEN-1 or pernicious anemia, and similar observations have been made both in transgenic hypergastrinemic INS-GAS mice and in gastrin-knockout mice treated with exogenous gastrin[32].

AGS-G$_R$ cells have again been used to investigate the mechanisms responsible for the effects of gastrin upon cellular migration and invasion. In response to gastrin stimulation, AGS-G$_R$ cells undergo a morphological

change, with the induction of a branched phenotype, coupled with extensive remodelling of the cell's actin cytoskeleton. These effects were abrogated by treatment with a CCK-2 receptor antagonist and the alteration in morphology was not seen when the parent AGS cell line was exposed to gastrin. In addition to the morphological changes observed in this cell line, there was also evidence of a change in migration which was again mediated *via* a CCK-2 receptor-dependent pathway. AGS-GR cells, but not AGS cells showed increased migration when cultured in the presence of amidated gastrin and experiments involving the co-culture of AGS-GR and AGS-GFP cells demonstrated that the effects of gastrin on cell migration were at least in part due to paracrine signalling[33].

INSIGHTS FROM TRANSGENIC ANIMAL MODELS

Generation of a number of transgenic mouse strains over the last 15 years has greatly facilitated understanding of the mechanisms by which members of the gastrin family of peptides regulate the processes involved in gastric epithelial carcinogenesis (summarised in Table 1).

Hypergastrinemic mouse models

HGAS: hGas mice transgenically overexpress a complete human gastrin mini gene including the gastrin promoter region in some liver cells. Because the enzymes required for the post-translational processing of preprogastrin are not present in this tissue, hGas mice selectively overexpress human progastrin[4]. These mice show increased colonic proliferation and increased susceptibility to colonic carcinogenesis[4]. However, in contrast to the INS-GAS mice described below, there is no overt gastric phenotype, and no alterations were observed in gastric proliferation[4] or radiation-induced apoptosis[23] in comparison to wild-type. This suggests that the effects of the gastrin family of peptides on gastric mucosa are predominantly due to amidated forms of the hormone[4].

INS-GAS: In contrast to the liver, neuroendocrine cells, including those present in pancreatic islets, possess the appropriate enzymatic machinery to allow processing of progastrin into glycine extended and amidated forms of the hormone. Transgenic INS-GAS mice were created by expressing a human gastrin minigene spliced onto the insulin promoter and this resulted in expression of the gene in the pancreatic islets of adult animals. INS-GAS mice therefore have elevated serum concentrations of amidated gastrin[34]. At a young age, these mice have a two-fold increase in plasma gastrin levels compared to wild-type, show increased numbers of gastric parietal cells and ECL cells and secrete up to twice the amount of acid[4]. Beyond 5 mo of age however, there are progressive changes in gastric histology and physiology in these animals, with a reduction in acid secretion, such that at 12 mo they secrete less acid than wild-type controls, and by 20 mo they are essentially achlorhydric. This is associated with a progressive loss of gastric parietal cells and ECL cells over the same time period. Concomitant with these changes in the oxyntic mucosa, there is macroscopic evidence of hypertrophy in the gastric fundus and histological evidence of intestinal type metaplasia, a histological entity that is widely recognized as being premalignant. By 20 mo of age, there is evidence of gastric dysplasia in 100% and frank malignancy in 75% of mice. In comparison, wild-type FVB/N mice maintained under similar conditions did not develop metaplasia, dysplasia or gastric carcinoma[19].

Whilst INS-GAS mice developed gastric malignancies spontaneously over 2 years, it was also shown that this process was accelerated significantly by *H felis* infection. After 6 mo, there was significantly more gastric atrophy in all *H felis*-infected INS-GAS mice relative to both uninfected INS-GAS and *H felis*-infected FVB/N mice. In addition, there was also evidence of malignant progression, with 85% of infected INS-GAS mice being reported as showing at least intramucosal carcinoma after 6 mo of infection, in comparison to 12.5% of the uninfected INS-GAS group and none of the FVB/N groups[19]. Subsequent investigations have demonstrated that *H pylori* as well as *H felis* can induce gastric cancer in these mice, however there was a significant difference in susceptibility between male and female INS-GAS mice. After 7 mo of infection, four of 12 infected males were found to have gastric adenocarcinoma, whilst none of the other groups developed malignancies. In this study, progression to metaplasia and dysplasia was observed in all groups, but more severe changes were present in male than female animals[35].

The *H felis*-infected INS-GAS model has also been used to investigate the effects of administering the CCK-2 receptor inhibitor YF476. When this drug, in conjunction with the H2 receptor antagonist loxtidine, was given to INS-GAS mice infected with *H felis*, it significantly inhibited the development of gastric atrophy, dysplasia, and adenocarcinoma[36].

MTI/G-Gly and INS-GAS/MTI/G-gly: A third type of transgenic mouse that produces glycine extended gastrin has also been created by inserting two stop codons into the human gastrin gene after glycine-72. This transgene was spliced with the mouse metallothionein promoter to create transgenic animals that express the transgene in all tested tissue types and which constitutively overexpress glycine extended forms of gastrin[5]. This mouse model does not demonstrate a gastric phenotype histologically, and gastric tumors are not seen in the mice at 1 year of age[5]. In order to investigate whether glycine extended forms of gastrin modulated the effects of amidated gastrin upon the stomach, INS-GAS and MTI/G-Gly mice were crossed to generate doubly transgenic INS-GAS/MTI/G-Gly mice. These mice demonstrated less mucosal atrophy than INS-GAS mice and there was evidence of acid hypersecretion rather than the hypochlorhydria observed in INS-GAS mice. The altered phenotype of

Table 1 Phenotype of mice with transgenic alterations in members of the gastrin family of peptides

Transgenic strain	Transgenic abnormality	Gastric phenotype	Susceptibility to gastric carcinoma	Other relevant phenotype
hGAS[4]	Human gastrin minigene expressed in liver-resulting in elevated serum levels of human progastrin	No known gastric phenotype	Not altered	Increased colonic mucosal proliferation[4,113] and susceptibility to azoxymethane-induced colon cancer[114]
MTI/G-Gly[5]	Human gastrin gene with two stop codons after glycine-72, spliced with MTI promoter. Transgenic animals have elevated serum levels of glycine extended gastrin	No known gastric phenotype	Not altered	Increased colonic mucosal proliferation[5]
INS-GAS[34]	Human gastrin minigene spliced with insulin promoter expressed in pancreatic islets-resulting in elevated serum levels of amidated gastrin	Initial gastric mucosal hypertrophy and excess gastric acid secretion. By 5 mo, gastric atrophy and hypochlorhydria. Increased gastric proliferation and increased susceptibility to apoptosis	Increased (spontaneous tumors at 20 mo and *H Felis*-induced tumors at 6 mo)	Increased colonic mucosal proliferation in proximal and distal colon but not rectum initially observed in 1-year-old animals[4], but no difference in apoptotic or mitotic rates seen in 10-12-wk-old mice[113] and no increase in AOM-induced cancers[114]
INS-GAS/ MTI/G-Gly[37]	MTI/G-Gly mice crossed with INS-Gas mice to result in a "double" transgenic mouse that expresses both increased amidated and glycine extended forms of gastrin	Hyperchlorhydric at birth but unlike INS-GAS, no mucosal atrophy at older ages. Reduced apoptosis compared to INS-GAS with similar levels of proliferation. Overall rates of malignant progression comparable to INS-GAS	Increased	
GAS-KO[38]	Gastrin knockout mice generated by targeted gene disruption	Achlorhydric with reduced parietal cell numbers (gastric atrophy), clustering of ECL cells at gland bases and increased TFF2-positive cells (spasmolytic polypeptide expressing metaplasia)	Increased	Increased susceptibility to azoxymethane-induced colon carcinogenesis[115] (despite normal untreated proliferation indices[113])
CCK-B-null[49]	Gastrin receptor knockout mice generated by targeted gene disruption	Marked gastric atrophy and achlorhydria. Morphologically abnormal ECL cells with loss of normal secretory vesicles and replacement with dense core granules and microvesicles	Not reported	Increased sensitivity to dopamine[116] and altered behaviour in response to alcohol[117,118] and other stimuli[119,120]

INS-GAS/MTI/G-Gly mice appeared to result from reduced gastric epithelial apoptosis rather than due to any changes in proliferation[37]. However, these changes in atrophy did not result in a reduction in malignant susceptibility, as at 18 mo all INS/GAS and all INS-GAS/MTI/G-Gly mice had developed gastric malignancies[37].

Gastrin-deficient mice

The consequences of gastrin deficiency *in vivo* have been investigated by a number of groups by generating gastrin knockout mice. Under normal animal house conditions, these animals develop gastric atrophy, with thinner gastric mucosae, fewer H^+/K^+ ATPase-positive parietal cells and impaired acid secretion[38,39]. In addition, the oxyntic mucosa contains fewer chromogranin A immunopositive ECL cells and increased numbers of TFF-2-expressing cells, indicative of spasmolytic peptide expressing metaplasia (SPEM)[40]. Also, ECL cells appear to be clustered towards the bottom of the gastric gland of gastrin knockout mice and there is a reduced rate of parietal cell migration to the base of the gland in these animals[41].

The gastric phenotype of gastrin knockout[42] mice predisposes these animals to colonisation of the stomach with bacteria[43], resulting in inflammation and an initial increase in parietal and G-cell numbers[44]. The long term effect of this chronic inflammatory state may be to promote malignant transformation, and in some laboratories gastric tumors have been found in gastrin knockout mice by 1 year of age[42,45]. When the specific effects of infection with *H pylori* strain 119/95 (a CagA positive, VacA positive strain previously shown to cause gastritis acutely[46], and gastric epithelial lymphomas with chronic infection in C57Bl/6 mice[47]) were investigated in these mice, there was an alteration in acid secretion, thought to be stimulated through a vagal response mechanism, but no increased risk of tumor development was observed at 6 mo[48].

Transgenic mice resistant to gastrin

CCK-2 receptor null mice have also been produced independently by two groups. These mice demonstrate

Table 2 Causes of hypergastrinemia in humans

Acidic gastric pH	Elevated gastric pH
Gastrinoma	Chronic atrophic gastritis
Antral predominant *H pylori* infection	Autoimmune
Pyloric obstruction	*H pylori* infection
Renal failure	Acid-suppressing medication
Retained gastric antrum following Billroth Ⅱ gastrectomy	Vagotomy

marked gastric atrophy and reduced acid secretion as predicted[49-51]. There is also evidence of morphological changes in ECL cells, resulting in cells with loss of normal secretory vesicles and replacement with microvesicles and dense core granules[52]. As far as we are aware, there are no reports of increased susceptibility to gastric carcinogenesis in CCK-2 null animals.

CAUSES OF HYPERGASTRINEMIA

Persistent hypergastrinemia can occur as a consequence of a number of different pathological states. These can broadly be divided into conditions which cause uncontrolled excess gastrin secretion such as gastrin-secreting tumors, and the normal physiological response to suppressed gastric acid secretion (Table 2). There is evidence that hypergastrinemia, particularly that which results from gastrinomas and chronic atrophic gastritis, may be associated with the development of gastric malignancies.

ZES

Neuroendocrine tumors of the pancreas and duodenum are rare, and are either functional, secreting one of a variety of neuropeptides, or non functioning, where elevated levels of neuropeptides are not detected. Of the functional tumors, the commonest hormone to be secreted is gastrin, accounting for up to 30% of such neoplasms. Gastrinomas have an incidence of 0.5-3 per million population per year[53] and were first described, along with a syndrome of gastric hypersecretion, in 1955 by Zollinger and Ellison[54]. The majority of gastrinomas are sporadic, however, approximately 12% are associated with the multiple endocrine neoplasia syndrome type 1 (MEN1)[55], in association with functional adenomas of the parathyroid (90%), pituitary [e.g. prolactinomas (17%)] and pancreas [e.g. insulinomas (10%)][56].

Unlike the first report by Zollinger and Ellison, in which one patient had radical surgery, but continued to secrete gastric acid and eventually died, and another required a total gastrectomy to control the adverse effects of gastric acid hypersecretion, today the prognosis for patients with gastrinoma is relatively good. The most important prognostic factor is the presence or absence of hepatic metastases. Patients without hepatic metastases at presentation (more than 75% of cases) have a 90%-100% 10-year survival, whilst those with metastatic disease have only a 10%-20% 10-year survival[53]. The advent of H2 receptor antagonists and subsequently PPIs has enabled control of gastric acid hypersecretion in the majority of patients, thereby reducing the risk of peptic ulceration. These therapies have made a significant impact upon the effects of ZES, however they target the consequences of hypergastrinemia rather than the underlying hormone production. Thus, surgery to remove the primary gastrinoma remains the only potentially curative option. This is feasible in at least 20%-45% of patients with sporadic ZES, but in far fewer patients with MEN1/ZES, as they are more likely to have multiple or diffuse tumors that are not amenable to surgery[53].

Helicobacter infection

H pylori infection is an important independent risk factor for gastric carcinogenesis and gastric atrophy. Observational data suggest that *H pylori* infection directly causes mild degrees of hypergastrinemia. For example, asymptomatic patients with *H pylori* colonisation have been shown to have elevated serum gastrin concentrations relative to a control population, despite similar gastric acid output[57], while Levi *et al*[58] demonstrated that following *H pylori* eradication, there was a reduction in fasting serum gastrin concentration. It has also been demonstrated that following eradication of *H pylori*, there was an increase in somatostatin mRNA and a concomitant decrease in gastrin mRNA in patients with duodenal ulcers. This was associated with increased numbers of D-cells in the gastric corpus[59], suggesting that the hypergastrinemia caused by *H pylori* infection may result from a loss of somatostatin control over gastrin secretion.

Atrophic gastritis

Gastric atrophy is defined as the loss of parietal cell mass, leading to decreased acid secretion and consequent increased luminal pH. This interrupts the somatostatin negative feedback mechanism and results in hypergastrinemia. The most important causes of gastric atrophy are autoimmune (associated with pernicious anemia) and chronic *H pylori* infection. Both types of atrophic gastritis are associated with hypergastrinemia, although the fasting serum gastrin concentration is usually more markedly elevated in the autoimmune type, due to the more profound loss of parietal cells.

Autoimmune atrophic gastritis: The epidemiology of autoimmune atrophic gastritis is similar to that of other autoimmune diseases, with a female to male predominance approaching 2:1 and with individuals in their 7th decade or later being typically affected[60]. This condition is characterised by vitamin B12 deficiency as a result of loss of intrinsic factor and is associated with autoantibodies towards gastric parietal cells and/or intrinsic factor. These autoantibodies are found in the sera of 70% and 55% of patients with pernicious anemia respectively and at least one autoantibody is present in 85% of patients. Although it has been demonstrated that these antibodies can be cytotoxic *in vitro*[61], it is less

Table 3 Types of gastric neuroendocrine tumor

Type	Associated diseases	Proportion of gastric NETs	Typical endoscopic findings	Plasma gastrin	Gastric juice pH	Prognosis
Ⅰ	Chronic autoimmune atrophic gastritis	80%	Multiple < 1 cm polyps	High	~7	Good
Ⅱ	ZES and MEN1	5%	Multiple < 1 cm polyps	High	< 2	Variable
Ⅲ	None	15%	Single 2-5 cm polyp	Unchanged	1-2	Poor

clear whether they are the responsible for causing gastric atrophy *in vivo*.

***H pylori*-associated atrophic gastritis:** The second important cause of gastric atrophy is chronic *H pylori* infection. Most commonly, primary infection occurs in childhood, hence many patients have long-term colonization of the stomach. This can have a number of consequences, ranging from increased gastric acid secretion and associated peptic ulcer disease to gastric atrophy with resultant increased luminal pH. The latter is associated with an increased risk of gastric cancer. The factors that influence the clinical outcomes of *H pylori* infection in individual patients remain poorly understood. The site of colonization within the stomach appears to be important however, with antral infections being particularly associated with peptic ulcer disease, whereas gastric corpus colonization is more likely to lead to gastric atrophy.

H pylori occupies a niche in the mucus layer of the gastric mucosa and produces urease enzymes which allow the pH of the immediate environment to be raised to physiological levels, thus facilitating prolonged colonization. Various bacterial, host and environmental factors have been suggested to influence the response to *H pylori* infection. For example, mouse models have demonstrated that polarization of the immunological response towards a Th1 type increases the risk of developing gastric atrophy and subsequent gastric cancer[62], while genetic studies have suggested that polymorphisms in immune-response genes may also influence the consequences of infection in human subjects[63].

H pylori is associated with gastric autoimmunity, although the specific mechanisms involved are not yet fully understood. Presotto *et al*[64] demonstrated that 58% of 79 asymptomatic patients with detectable anti-parietal cell antibodies had serological or histological evidence of *H pylori* infection compared to 39% of a control population ($P = 0.03$). It has also been proposed that early gastric autoimmunity may be reversible when concomitant *H pylori* infection is treated[65]. The precise interactions between *H pylori* infection and the development of autoimmune gastritis therefore warrant further investigation.

GASTRIC TUMORS ASSOCIATED WITH HYPERGASTRINEMIA

The hypergastrinemia which results from the causes described above is associated with increased risks of developing various different gastric tumors.

Hypergastrinemia in the absence of *H pylori* infection is most strongly associated with the development of gastric neuroendocrine tumors. In contrast, the hypergastrinemia associated with chronic *H pylori* infection may act as a co-factor during the development of gastric adenocarcinoma. In the following section, we will discuss the role of gastrin in the pathogenesis, diagnosis, and treatment of these specific gastric tumor types.

Gastric neuroendocrine tumors

Pathogenesis: Gastric neuroendocrine (carcinoid) tumors are the classical example of gastrin-induced malignancies. These neoplasms are derived from ECL cells, which are the most abundant neuroendocrine cell type in the oxyntic mucosa. Hypergastrinemia alone appears to be sufficient to induce ECL cell hyperplasia, however, for macroscopic neuroendocrine tumor formation, additional triggers are required. For example, lifelong therapy of rats with PPIs resulted in the development of ECL cell tumors[66], whereas the same has not been observed in humans despite induction of hypergastrinemia[13,14]. This suggests that additional host or environmental factors are required for tumor development.

The typical conditions in which ECL tumors develop are either gastric atrophy associated with pernicious anemia (type I gastric neuroendocrine tumors), or the presence of prolonged hypergastrinemia and mutation of the MEN1 gene in ZES associated with MEN type I (type Ⅱ gastric neuroendocrine tumors)[67] (Table 3). The latter condition provides further evidence that hypergastrinemia alone may be insufficient to cause gastric neuroendocrine tumors, as the relative risk for developing such neoplasms is at least 70 fold lower in patients with sporadic ZES compared with those who have ZES associated with MEN1[68].

In pernicious anemia, there is a reduction in the number of gastric parietal cells and subsequent achlorhydria. This affects the somatostatin feedback loop that controls gastrin secretion, thereby rendering the patient hypergastrinemic. Hypergastrinemia then provides a proliferative drive to ECL cells (Figure 3). The achlorhydric environment also provides opportunities for further microenvironmental changes, including the provision of a niche for bacterial colonization. Interest in this aspect of gastric carcinogenensis has been present since the 1980s, when numerous studies demonstrated increased levels of N-nitroso compounds (potential carcinogens that are metabolised by intra gastric bacteria from nitrosamines) in the gastric lumens of patients

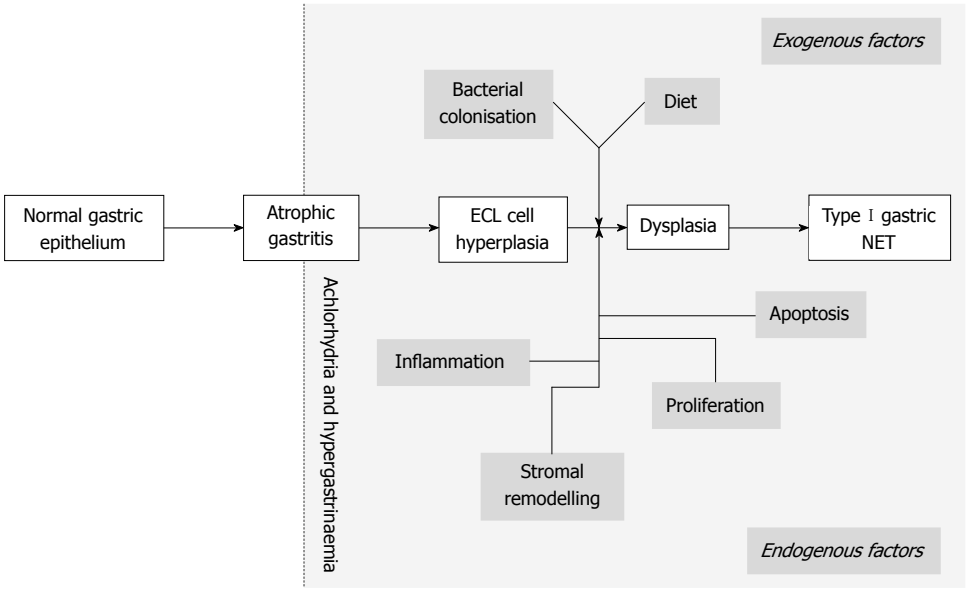

Figure 3 Pathway of development of type Ⅰ gastric neuroendocrine tumors.

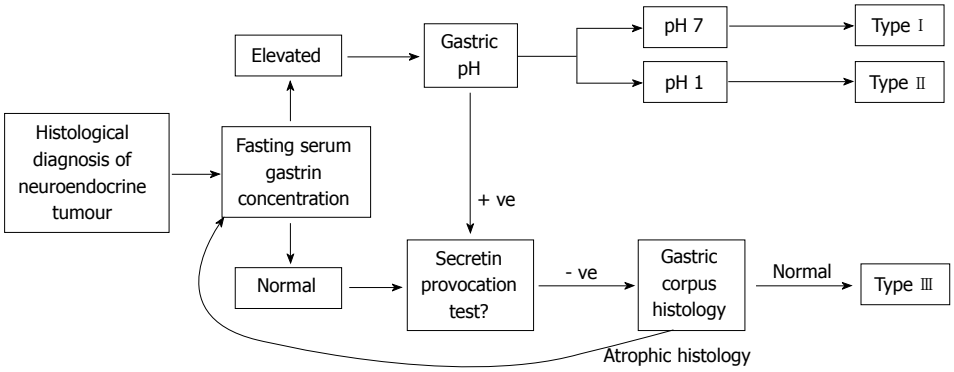

Figure 4 Diagnostic algorithm for gastric neuroendocrine tumors.

with both atrophic gastritis and gastric malignancy[69].

The major risk factor in addition to hypergastrinemia that promotes the development of type Ⅱ gastric neuroendocrine tumors is mutation of the *MEN1* gene. Gastric neuroendocrine tumors are seen in less than 1% of patients with sporadic ZES, whilst in those with ZES associated with MEN type Ⅰ, the prevalence is 13%-43%[70]. A recent prospective study of 57 consecutive hypergastrinemic MEN1 patients reported that 100% had abnormal ECL cell distribution and 23% had gastric neuroendocrine tumors.

The *MEN1* gene encodes a 610-amino-acid protein, menin. Menin is expressed in many diverse tissue types and is localized in the nucleus. It binds directly to DNA in a sequence-independent manner and is also capable of binding several other nuclear factors including transcription factors and DNA repair proteins. The physiological function of menin is as a tumor suppressor, although the specific mechanism of action is less clear. The mutations seen in the *MEN1* gene result in either reduced expression of menin or in some cases complete absence of menin[56].

Interestingly, local rather than somatic mutations of menin may also be of significance in the pathogenesis of type I gastric carcinoids and two studies have assessed loss of heterozygosity (LOH) of 11q13, the locus for the *MEN1* gene, in this tumor type. The smaller study assessed three gastric neuroendocrine cell tumors and demonstrated LOH at this locus in two patients and a localized mutation of *MEN1* in one[71]. The larger study investigated 17 type I gastric neuroendocrine tumors, four type Ⅲ gastric neuroendocrine tumors and two histologically defined neuroendocrine carcinomas. 47.1% of type I neuroendocrine tumors had LOH at 11q13 compared to 25% of the type Ⅲ neuroendocrine tumors, while both neuroendocrine carcinomas showed substantial deletions at this locus[72].

Diagnosis: Clearly, measurement of fasting serum gastrin concentration is an important component in the diagnostic pathway for patients with gastric neuroendocrine tumors. Once a histological diagnosis of gastric neuroendocrine tumor has been made, it is imperative to ascertain the type of tumor, as well as its stage, as this will influence treatment. Defining the type of tumor can be achieved in most cases by measuring the fasting serum gastrin concentration along with the pH of gastric juice (Figure 4). In the presence of a normal or low fasting serum gastrin concentration, a diagnosis of sporadic or type Ⅲ gastric neuroendocrine tumor is most likely. In the context of an elevated serum gastrin concentration, the gastric juice pH determines whether a type I lesion (neutral pH) or type Ⅱ lesion (acidic pH) is present. This simple algorithm provides

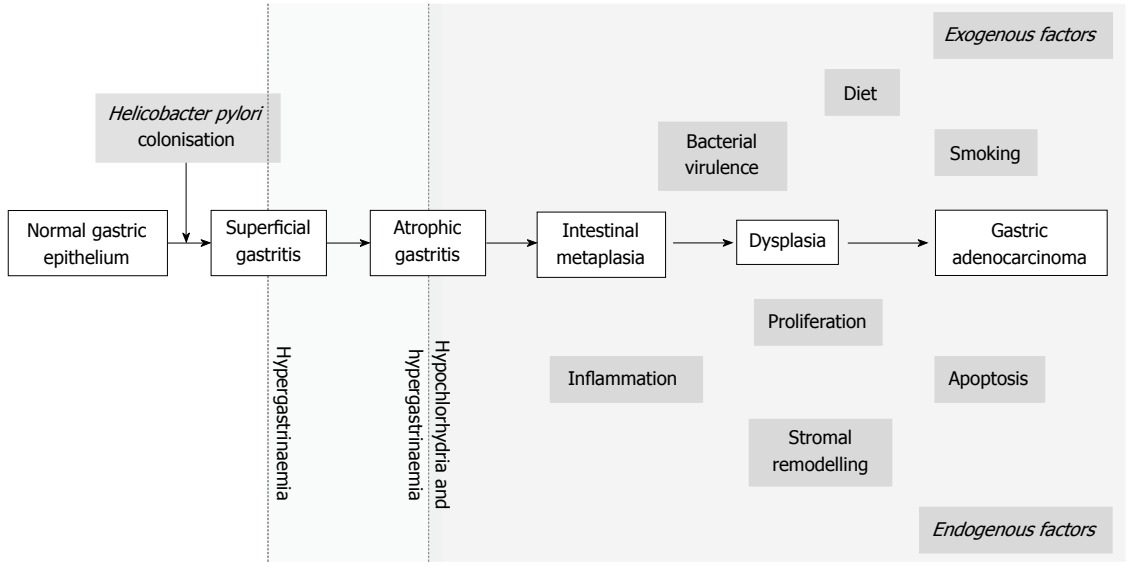

Figure 5 Pathway of development of gastric adenocarcinoma.

the initial data that inform future investigations. Many of these lesions are still gastrin sensitive at the time of diagnosis, and in such cases, removal of the source of hypergastrinemia by antrectomy (type Ⅰ) or gastrinoma resection (type Ⅱ) should result in tumor regression. To investigate whether a type Ⅰ gastric neuroendocrine tumor is gastrin sensitive, it may be useful to perform an octreotide suppression test. This involves biopsying the gastric corpus mucosa and neuroendocrine tumors pre and post octreotide infusion and assessing whether there is a reduction in mRNA abundance for the secretory components of ECL cells, such as histidine decarboxylase, following octreotide administration. This surrogate is then used as a marker for gastrin sensitivity[73].

Treatment: Those type Ⅰ and Ⅱ gastric neuroendocrine tumors that retain gastrin sensitivity may be amenable to treatment by methods which reduce elevated serum gastrin concentrations. This may involve locating and excising a primary gastrinoma in the case of a type Ⅱ gastric neuroendocrine tumor, or surgical antrectomy in a patient with pernicious anemia and autoimmune chronic atrophic gastritis. Very occasionally, large type Ⅰ gastric carcinoids grow autonomously and are no longer gastrin-sensitive, and such tumors may require gastrectomy. Future treatments for types 1 and 2 neuroendocrine tumors may include the use of CCK-2 receptor antagonists to inhibit the effects of elevated serum gastrin concentrations.

Gastric Adenocarcinoma

Pathogenesis: Gastric adenocarcinoma accounts for the greatest mortality and morbidity associated with primary malignancies of the gastric mucosa. It develops through a stereotypical pathological sequence[74] characterized by progression from chronic active gastritis to atrophic gastritis, and *via* metaplastic lesions to dysplasia and malignancy (Figure 5). As described above, gastrin secretion is altered following *Helicobacter* infection. There is accumulating evidence, particularly from the INS-GAS animal model described above, that hypergastrinemia may contribute towards the development of gastric atrophy and remodelling of the gastric mucosa. However, it is currently not clear to what extent inferences about human gastric carcinogenesis can be made from the observations in these transgenic mice.

Diagnosis: Measurement of fasting serum gastrin concentration is reasonably simple and therefore several groups have investigated whether such assessment assists in the diagnosis or helps to determine the management of gastric cancer. Although a statistically significant elevation in serum gastrin concentration has been demonstrated in patients with gastric cancer compared to controls[75,76], the clinical usefulness of such assessment is currently limited. Attempts to use fasting serum gastrin concentration either as a marker of prognosis and resectability[77] or as a part of a panel of surrogate markers for initial diagnosis[78] have also been unsuccessful, and such approaches have largely been superceded by advancements in endoscopy and imaging techniques.

More recently, assessment of serum gastrin concentration has been evaluated as part of a panel of potential biomarkers for determining the presence and location of gastric atrophy. A Swedish population of 1000 individuals between the ages of 20-80 years underwent gastroscopy with biopsies taken from the antrum, corpus and fundus to assess for the presence of atrophy and at the same time had blood taken for analysis of gastrin-17, *H pylori* IgG antibody, pepsinogen Ⅰ and Ⅱ. This panel had a relatively high false positive result of 31%, although the authors defended this finding by emphasizing the potential pitfalls of sampling error when relying on histological assessment of small mucosal biopsies. Indeed, they asserted that the finding of serological markers in keeping with atrophy in

4.9% of patients with histological evidence of *H pylori*-associated non-atrophic gastritis, compared to just 0.8% of *H pylori*-negative patients who had no histological evidence of atrophy, potentially demonstrates the strength of serology as a global marker of atrophy rather than being limited to samples obtained from a small geographic area. Taking histology as the gold standard, these biomarkers diagnosed corpus atrophy with a positive predictive value of 69% (CI 95%: 66-72%) and a negative predictive value of 98% (95% CI: 97-99%) in this population[79].

Hansen *et al*[80] recently investigated a cohort of 101 601 patients from whom serum samples were collected in Sweden in the 1970s. They compared 230 patients who developed gastric cancer by 1992 with controls from the same cohort, and demonstrated a significant correlation between elevated serum gastrin concentration and low serum pepsinogen Ⅰ/Ⅱ ratios, and increased risk of developing non-cardia gastric cancer. They also demonstrated that most cases of gastric cardia cancer were not associated with *Helicobacter* infection, and did not show an association with markers of gastric atrophy; however, a minority of cases had serological evidence of past or present *Helicobacter* infection and an association with markers of gastric atrophy. This suggests that gastric cardia cancers develop most commonly through a *Helicobacter*/gastrin-independent mechanism, with a small subset having a similar etiology to non-cardia cancers.

The same panel of biomarkers have also been assessed in other cohorts, including a pediatric population, but in this case, they were found to be insufficiently sensitive to diagnose *Helicobacter* infection or the low incidence of atrophy that is present in this population[81]. Endoscopic evaluation of the upper GI tract therefore remains the accepted gold standard for the assessment of mucosal lesions of the GI tract; however, there may be a role, which needs further investigation, for a panel of biomarkers such as those described above in identifying adults at particularly high risk of gastric atrophy.

Treatment: Various groups have assessed whether immunohistochemical analysis of gastric carcinoma tissue for the presence of gastrin and/or the CCK-2 receptor is useful for predicting prognosis. Normal gastric mucosa expresses the CCK-2 receptor on ECL cells and parietal cells. Immunohistochemical studies using archival samples have shown that as the Correa sequence progresses, with development of gastric atrophy and parietal cell loss, an increased percentage of cells in the gastric corpus express the CCK-2 receptor[82]. In normal gastric corpus mucosa, gastrin immunopositive cells are not detectable; however, with progression down the Correa sequence, increased expression of gastrin and its precursors have been observed[82]. An immunohistochemical study based on a tissue array of 304 gastric cancer resection specimens from Korea demonstrated that 56.5% expressed CCK-2 receptors within the malignant tissue and that gastrin was detectable within the tumor mass in 47.7%[83]. A Welsh study has also demonstrated adverse survival in patients whose gastric tumors stained positively for gastrin[84]. These observations suggest that gastrin may represent a potential therapeutic target for the prevention or treatment of gastric carcinoma. Initial attempts at targeting gastrin to improve survival in gastric cancer used proglumide, a weak CCK-2 receptor inhibitor, and a randomized controlled trial demonstrated no survival benefit following treatment with this drug[85]. Subsequent developments have included the development of G17DT, an immunogenic mimic of gastrin 17 that causes the production of anti-gastrin antibodies[86]. Immunization with this agent improved the survival of severe combined immune deficient (SCID) mice xenografted with the human gastric cancer cell line, MGLVA1asc, and this effect was equivalent to combination chemotherapy with 5-FU and leucovorin. Moreover, the use of 5-FU in conjunction with G17DT appeared to have an additive effect[87]. G17DT was well tolerated in phase Ⅱ clinical trials[88], hence a multicenter phase Ⅱ trial of G17DT in conjunction with 5-FU and cisplatin in advanced gastric cancer has recently been performed. 60% of G17DT treated patients successfully developed anti-gastrin-17 antibodies and this subgroup showed significantly improved survival[89].

Gastric mucosa-associated lymphoid tissue (MALT) lymphomas

MALT lymphomas are marginal zone lymphomas derived from the mucosa-associated lymphoid tissue of the stomach. They occur in patients with gastric atrophy and are strongly associated with *H pylori* infection. MALT lymphomas are associated with both hypergastrinemia and overexpression of the CCK-2 receptor in the gastric mucosa. The presence of hypergastrinemia is not surprising in view of the association with gastric atrophy, but the overexpression of CCK-2 receptor suggests a potential mechanism through which gastrin may exert a trophic effect in this tumor type[90,91].

Fundic cystic gland polyps

These lesions are found in the oxyntic mucosa and are described by the World Health Organization as a proliferation of surface foveolar cells lining elongated, distorted pits that extend deep into the stroma. They show hyperplasia of mucous neck cells and variable amounts of cystic dilatation[92]. Although they are not intrinsically dysplastic, progression to gastric malignancy has been reported, particularly when there is underlying familial adenomatous polyposis or juvenile polyposis. Patients with these conditions have fundic cystic gland polyps on endoscopy in up to 90%[93] of cases and of these polyps, up to 40%-50%[93,94] have associated dysplasia.

Patients without inherited polyposis syndromes, however, account for the vast majority of those with fundic cystic gland polyps. In this group, the association between polyps and dysplasia is far less clear cut. Although dysplasia and cancers have been reported in patients who have these lesions[95-97], it is not clear

whether the incidence of malignancy is significantly higher than that of the normal population.

The association between fundic gland polyps and gastrin is derived from the recognition that these lesions occur more commonly in patients who are taking long-term PPIs. However, PPI-induced fundic cystic gland polyps probably have a different etiology compared to the sporadic polyps that occur in patients who are not taking PPIs (which are associated with somatic mutations in β-catenin[92,96,97]) and the polyps associated with FAP or juvenile polyposis, where there are known molecular aberrations. It has been suggested that PPI-induced fundic cystic gland polyps arise as a result of impaired glandular flow after hypergastrinemia-induced parietal cell hyperplasia has caused a mechanical obstruction to the gland[98]. However, this does not appear to be related to the severity of hypergastrinemia, as a small Norwegian study has shown equivalent degrees of hypergastrinemia in patients taking PPIs with and without fundic gland polyps[99].

In the context of PPI usage, the risk of malignant transformation is extremely low and there is at present no recommendation either for endoscopic removal of lesions or for any form of endoscopic surveillance in patients with fundic cystic gland polyps who are taking PPIs, unless they also have a familial polyposis syndrome[98].

DISCUSSION

Evidence therefore suggests that gastrin may affect the risk of developing various epithelial and possibly lymphoid gastric malignancies by altering key cellular pathways including proliferation, apoptosis, migration, tissue remodelling and possibly angiogenesis. Gastrin is also a potential therapeutic target for the treatment of various gastric tumors. For example, surgical approaches to correct hypergastrinemia may be employed for some gastric neuroendocrine tumors, while agents such as G17DT have shown some promise in phase Ⅱ clinical trials in advanced gastric adenocarcinoma[89]. Such approaches may also have future uses in the prevention of malignancy, for example in patients who have precursor lesions such as ECL cell hyperplasia or gastric atrophy. If such therapies prove effective, there may additionally be a need to reconsider the role of endoscopic surveillance for gastric atrophy, an approach that has lost favor recently.

In population terms, more people are hypergastrinemic than ever before as a result of continued increases in the prescription rates of PPI drugs. In comparison to the numbers of individuals prescribed these drugs, gastrin-associated malignancies are undoubtedly rare, however, there is an ongoing debate about the safety profile of these agents[98,100-102].

Animal studies have shown that ECL cell hyperplasia can occur in response to hypergastrinemia induced by chronic proton pump inhibition[12]. In rats that were rendered achlorhydric for their entire lifespan, ECL-cell-derived neuroendocrine tumors also developed[66]. However, there is no convincing evidence that PPIs cause ECL cell malignancies in humans, possibly because PPIs do not usually induce complete achlorhydria[103]. Although no tumors have been found, there is evidence of diffuse and linear patterns of ECL cell hyperplasia in patients treated for a decade with PPIs[13-14,104].

There has also been concern about whether PPI treatment modulates the consequences of chronic *H pylori* infection. Recent studies have compared patients treated with anti-reflux surgery and those treated with PPIs. In patients who were *H pylori*-negative, treatment for 7 years with a PPI made no difference to mucosal inflammation or atrophy. However, patients who were *H pylori*-positive over the same 7-year period showed increased progression towards mucosal atrophy and increased inflammation if treated with a PPI, in comparison to those treated with surgery[104]. Although this suggests that *H pylori* infection should be eradicated before initiating chronic acid suppression therapy, the observed changes were modest and to date there has been no evidence of progression beyond mucosal atrophy. The studies were not designed to assess whether there was any associated increase in the incidence of gastric cancer, and much larger cohorts would be required to investigate this. Epidemiological database studies have been cited as demonstrating that PPI prescription is associated with an increased risk of gastric cancer[98]. However these data remain unpublished and this type of retrospective analysis cannot reliably distinguish patients who have been prescribed PPIs for the presenting symptoms of gastric cancer from any increase in the incidence of gastric carcinoma as a result of PPI use.

Current evidence therefore suggests that the relatively modest hypergastrinemia induced by PPI drugs is not associated with malignant transformation in the human stomach. When considering the overall safety profile of this class of drugs, gastrin-independent adverse effects such as malabsorption of vitamin B12[105,106] and iron[107], susceptibility to bacterial infection[108-110] and osteoporosis[111,112] should also be considered. Although CCK-2 receptor inhibition or combined PPI and CCK-2 receptor inhibition have been suggested as potential ways of reducing the gastrin-mediated side effects of PPIs, it seems unlikely that this approach will be clinically useful whilst PPIs have such a good safety profile, and especially as some of the adverse effects are probably related to hypochlorhydria rather than hypergastrinemia.

Our understanding of the importance of gastrin in gastric tumorigenesis has therefore increased significantly over recent years and the generation of transgenic animal models has greatly facilitated our understanding of the mechanisms involved. Issues that still need clarification include the precise role of gastrin in the pathogenesis of human gastric adenocarcinoma, whether pharmacological targeting of gastrin or its receptor is beneficial for the treatment and/or prevention of various gastric tumors and whether PPI-induced hypergastrinemia has any long term clinically important consequences, particularly in the context of chronic *H pylori* infection.

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Eosinophilic esophagitis

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Abstract

Eosinophilic esophagitis is increasingly recognized in adults. The diagnosis is based on the presence of both typical symptoms and pathologic findings on esophageal biopsy. Patients usually present with dysphagia, food impaction and/or reflux-like symptoms, and biopsy of the esophagus shows more than 15 eosinophils per high-power field. In addition, it is essential to exclude the presence of known causes of tissue eosinophilia such as gastroesophageal reflux disease, infections, malignancy, collagen vascular diseases, hypersensitivity, and inflammatory bowel disease. There are no standardized protocols for the therapy of eosinophilic esophagitis. A variety of therapeutic approaches including acid suppression, dietary modifications, topical corticosteroids and endoscopic dilation can be used alone or in combination.

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Key words: Eosinophilic esophagitis; Dysphagia; Endoscopic dilation; Reflux; Gastro esophageal reflux disease

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INTRODUCTION

Eosinophilic esophagitis (EE) in adults is a disease with the following clinicopathological characteristics: (1) symptoms including but not restricted to food impaction and dysphagia; (2) biopsy specimen showing more than 15 eosinophils/high-power field (HPF); and (3) other disorders associated with similar clinical, histological, or endoscopic features have been excluded[1]. EE is increasingly being recognized in adult and pediatric populations, either as a separate entity or as a part of the spectrum of eosinophilic gastroenteritis[2]. It was initially described in the 1970s[3], but subsequently most research focused on gastroesophageal reflux disease (GERD) as being the primary cause of esophageal eosinophilia. It was not until the 1990s that EE came to be regarded as a separate entity[4]. Later on, an allergic component to EE was observed as patients suspected of having EE had improvement of symptoms on either elemental diet[5,6] or on corticosteroids[7]. These features, along with normal pH study results and the relative lack of effectiveness of acid suppression therapy, resulted in EE being regarded as a clinical condition different from GERD.

EE has recently been explored in much detail within various forums. This article aims to review that literature looking at the epidemiological and clinicopathological aspects of EE, with special emphasis on diagnostic approaches and treatment options.

EPIDEMIOLOGY, ETIOLOGY AND PATHOGENESIS

EE has been studied most extensively in pediatric populations and only recently has further data been compiled in adults. EE can present in the third and fourth decades of life and various studies implicate it to be more predominant in men[8,9]. Among different races and ethnic groups, EE has been seen to be more prevalent in the white population[10]. Geographic distribution is wide, with cases now being reported not only in the United States but also Europe, Canada, Brazil, Japan and Australia[11]. The preponderance of EE in developed nations has unclear etiology and could

either be secondary to the increased prevalence of atopic diseases like asthma[12] or simply because of better reporting and data collection. To probe this further, Cherian *et al*[13] conducted a blind retrospective study of western Australian children investigated for esophageal disease in 1995, 1999 and 2004 and found the prevalence of EE to be indeed increased by 18-fold during this time period. The most recent US study in 74 162 patients used a national pathology database of subjects undergoing upper endoscopy with biopsy. The data confirmed that EE is a male-predominant disorder (74%) and that it can occur at any age. Over the study period (2002-2006), an increasing prevalence was noted. Whether this reflects a true increase in prevalence or increased recognition due to heightened awareness among physicians remains to be determined[14].

Furthermore, because of the past difficulty in diagnosing EE correctly in populations with dysphagia, food impactions or GERD, studies were subsequently conducted in various nations that later found EE to be the primary cause of these symptoms[7,15]. Markowitz *et al*[6] found that 15% of patients initially suspected of having GERD were actually discovered to have EE. They used strict diagnostic criteria for EE such as > 20 eosinophils/HPF in esophageal biopsies, normal esophageal pH monitoring and a lack of response to proton pump inhibitor (PPI) therapy. These findings may warrant changing our diagnostic approach to having an early esophagogastroduodenoscopy (EGD) with subsequent esophageal biopsies if clinical reflux-like symptoms and lack of response to medical treatment continue to be an issue.

Allergens play an important role in the etiology of EE. Kelly *et al*[5] first showed the association of food allergens with EE when they fed an elemental diet to 10 children with unremitting reflux symptoms and found symptomatic and histological improvement. This was later supported in different studies with successful use of either an elemental or a six-food elimination diet[16]. Aero-allergens form another potential cause of EE. Mishra *et al*[17] used a murine model to demonstrate an etiological role for inhaled allergens and eosinophils in gastrointestinal inflammation. A high degree of atopy and polysensitization to several environmental allergens was recently documented in patients with EE, suggesting that sensitization may partly be a response to inhaled allergens[18]. However multiple etiological factors are not mutually exclusive, as was presented by Plaza-Martin *et al*[19] who found evidence of poly-sensitization to aero-allergens and food allergens in their study population of patients who had EE. A familial pattern of inheritance has also been suggested to play a role in the development of EE[20].

The esophagus is normally devoid of eosinophils, however the rest of the gastrointestinal tract is populated with eosinophils beginning from the embryonal stage. Mishra *et al*[21] showed that the peptide eotaxin regulates eosinophil homing to the gastrointestinal (GI) tract during embryonal development. They also showed a connection between allergic hypersensitivity response in lung and esophagus regulated by eotaxin and interleukin (IL)-5[17,22]. It was subsequently shown that IL-13 plays a fundamental role in EE[23]. Once eosinophils have migrated to the esophagus, they release chemoattractants IL-3, IL-5 and granulocyte monocyte-colony stimulating factor (GM-CSF)[24]. Straumann *et al*[25] further confirmed the allergic nature of EE when they showed that a TH2 response, IL-5 and IgE mediated the pathogenesis of EE. However, there are inconsistencies in determining the exact nature and influence of an aero-allergic etiopathogenesis of EE. Balatsinou *et al*[26] observed EE in two patients with anticonvulsant hypersensitivity syndrome, wherein they saw reversal of endoscopic appearance after stopping carbamazepine, suggesting that oral agents could also play a role in the pathogenesis. An association with pollen has also been noted previously[27].

Dysphagia predominantly seen in EE has been attributed to both organic and non-organic (i.e. motility) disorders. Stevoff *et al*[28], in one of the first case reports on EE in octogenarians, showed circumferential but asymmetric thickening of the muscularis propria or a functional constriction related to myenteric plexus infiltration. Various other factors have been implicated in the development of dysphagia. Non-anatomic causes for dysphagia could be related to dysmotility. Nurko *et al*[29] reviewed the different causes of dysmotility that had been proposed in earlier papers, and these included eosinophil-mediated increased contraction of fibroblasts, axonal necrosis or cholinergic pathway interference, all of which contributed to esophageal dysmotility. A caveat to some of these studies is that they were either based on non-allergic models of esophagus or from studies in organ systems other than the esophagus[29].

CLINICAL FEATURES

EE usually presents with a multitude of symptoms, in part because it is a chronic disease and partly because of the gradual inflammatory involvement of the mucosa and submucosa before symptoms develop[30]. It can, however, present acutely as seen in food impactions[30]. The most common presenting symptom is dysphagia[30,31] but other symptoms such as nausea, vomiting, heartburn, chest pain or abdominal pain can also occur. Symptoms suggestive of esophageal dysmotility may indicate involvement of the muscular layers of the esophagus[32]. Occasionally, presentation of EE has been seen to be more subtle as patients adapt their chewing habits, eating food more slowly and washing down solid food with liquids, thereby decreasing the symptom incidence and leading to a delay in diagnosis[33]. Remedios *et al*[31] found an association of esophageal symptoms with exposure to certain foods even without actual consumption. Not infrequently, patients may also have additional symptoms of asthma[3], allergies or atopic dermatitis. Dauer *et al*[34] and Orenstein *et al*[35] have reported nasal symptoms and rhinosinusitis in about a quarter of patients that have EE. Laryngeal symptoms include hoarseness, cough, croup and sleep-disordered breathing[36]. Ferguson and

Table 1 Clinical presentation of eosinophilic esophagitis

Gastrointestinal symptoms	Other symptoms
Dysphagia	Chest pain
Food impaction	Rhinitis
Nausea and vomiting	Asthma
Heartburn	Allergies
Abdominal pain	Atopic dermatitis
Feeding disorders (pediatric)	Hoarseness
Failure to thrive (pediatric)	Croup, cough
	Sleep disordered breathing

Table 2 Clinical signs in eosinophilic esophagitis

Endoscopic features	Histologic features
Diminished vascular pattern	Thick epithelium with eosinophilia
Mucosal furrows	Abnormally long papillae
Thick mucosa	Fibrotic lamina propria
Exudates	Microabscesses
Strictures	Extracellular Eosinophilic granules
Rings	Increased extracellular major basic protein (MBP)
Laryngeal edema, vocal cord nodules, laryngeal ventricular obliteration	

Foxx-Orenstein divided the clinical manifestations according to age groups. Thus feeding disorders and failure to thrive were primarily seen in children below 2 years of age; vomiting, abdominal pain and reflux were seen in pediatric populations up to the age of 12; whereas adults usually present with dysphagia and food impactions[37] (Table 1).

Endoscopically, a normal-appearing esophagus is usually incompatible with a diagnosis of EE, although the findings can be subtle[8]. Typical findings on an EGD that imply the presence of EE include attenuation of subepithelial vascular pattern[38], linear furrowing[39] that may extend along the whole length of the esophagus, surface exudates composed of eosinophils or abscesses or strictures[8]. Presence of mucosal changes suggestive of ulcerations usually implies peptic injury by itself or in association with EE[40]. Schatzki ring has also been previously associated with EE[41] but one of the most characteristic and frequently quoted patterns is that of stacked circular rings or felinization[40], so called because of their presence in the cat esophagus. This has been postulated to be due to lamina propria and dermal papillary fibrosis caused by either the mediators that stimulate eosinophils or through the effect of eosinophils themselves. Previously, Vasilopoulos and Shaker described a small caliber esophagus as a major cause of dysphagia in patients with EE[42]. The esophagus was seen to have a smooth, diffusely narrow lumen shown on barium esophogram or esophagoscopy. Food impactions are also relatively frequent in patients with EE. Fox *et al*[40] have attributed these food impactions to either the strictures themselves or to decreased peristalsis secondary to underlying inflammation. Therefore, in patients with food impaction, it is worthwhile to follow EGD with biopsies for early diagnosis and treatment of this disorder. Airway endoscopy findings in patients with recurrent croup and EE include diffuse laryngeal edema, vocal fold nodules and laryngeal ventricular obliteration[36] (Table 2).

Histopathologically, EE is characterized by the presence of a thick epithelium with a large number of intraepithelial eosinophils lined near the surface, abnormally long papillae and a fibrotic lamina propria containing eosinophils[43]. Cheung *et al*[44], in their retrospective study of 42 children with dysphagia, described the presence of extracellular eosinophilic granules in patients with EE. Major basic protein (MBP) is a byproduct of eosinophil degranulation, and as such, increased deposition of MBP has been observed in pediatric and adult patients with EE[45,46].

Complications arising from EE can either be attributed to the clinical manifestations of the disease itself or to diagnostic and therapeutic interventions. Acute food impaction is one of the main reasons patients present as emergencies to the hospital. In one study, 57% of patients with EE had strictures that were successfully treated with dilatation, with subsequent resolution of symptoms[8]. More severe disease could lead to long segment narrowing which has been postulated to be in two forms[47]. The first form is referred to as trachealization[48], corrugated esophagus[48,49] or feline esophagus[50]. The second form is the small-caliber esophagus mentioned by Vasilopoulos *et al*[51], who found a diffusely narrow esophagus in three out of the five patients referred to them for chronic dysphagia. EE may also predispose to fungal and viral infections in the absence of steroid treatment or immunosuppression[47]. Straumann *et al*[52] conducted a chart review of 251 cases of esophagitis and found a case of Boerhaave's syndrome (spontaneous esophageal rupture). In their report, they recommend that all Boerhaave's cases be evaluated for EE. Chronic inflammation in EE may also lead to dysfunction of the lower esophageal sphincter and cause secondary reflux disease, as was reported by Remedios *et al*[31].

The risk of esophageal perforation is significantly increased during diagnostic or therapeutic endoscopic evaluation in a patient with EE[50]. In their chart and pathology review, Kaplan *et al*[50] found that more than half of their patients with EE had mucosal rents after simple passage of the endoscope, with one patient developing a perforation. Therefore, intense retrosternal pain after endoscopic evaluation in a patient suspected to have EE should particularly raise the suspicion of perforation and appropriate diagnostic evaluation should be undertaken[53]. The esophageal mucosa in EE is very fragile and inelastic, which Straumann *et al*[54] have termed "crepe paper mucosa" as it tore easily even with minor trauma. Consequently therapeutic interventions such as food bolus removal, dilation or biopsy can pose an even higher risk of perforation[52,55,56]. Kaplan *et al*[50] recommend about 8 wk of medical therapy before considering dilation in patients diagnosed with EE because of the high risk of perforation and the good response to medical therapy (Table 3).

Table 3 Complications in EE related to disease and to the interventions performed for treatment

Complications of EE	Complications of therapeutic interventions
Acute food impactions	Mucosal rents/tears
Long and short segment narrowing	Perforation
Stenosis	Infections-due to chronic use of steroids
GERD	Nutritional deficiencies
Boerhaave's syndrome	
Nutritional deficiencies	

DIAGNOSIS

EE should always be considered in the following circumstances: (1) history of food impaction; (2) persistent dysphagia especially in young individuals and in patients having a history of atopy; or (3) GERD refractory to medical therapy. Other causes of eosinophilia such as parasitic infestations, malignancy, drug hypersensitivity, collagen vascular diseases and inflammatory bowel disease need to be ruled out[57]. EE is primarily a clinicopathological condition and hence both symptoms and pathological diagnosis form an integral part of the diagnosis. The First International Gastrointestinal Eosinophilic Research Symposium (FIGERS) came up with comprehensive guidelines regarding the diagnostic criteria for EE. Accordingly, an eosinophil count of $\geqslant$ 15/HPF, along with normal gastric and duodenal biopsies, can substantiate the diagnosis of EE. Moreover, patients must have biopsies after 6-8 wk of twice daily acid suppression with PPI or have a negative pH study result[1] in order to correctly diagnose EE. At least five such biopsies must be obtained and preferably from both the proximal and the distal esophagus to account for the heterogeneous nature of the tissue eosinophilia[31,58] (Table 4).

Prasad *et al*[59] did a prospective study of 376 patients and found that mid-esophageal biopsies had a yield rate of about 10%. They thus recommend taking mid-esophageal biopsies in patients with unexplained food dysphagia. However, EE is patchy and hence increasing the number of biopsy specimens would theoretically yield a higher sensitivity and specificity in diagnosing EE[58]. Gonsalves conducted a chart review of 76 patients and found that sensitivity increased from 55% to 100% if the number of biopsies were increased from one to about five[58]. No statistically significant difference was found between the biopsies obtained from either the proximal or the distal esophagus. Thus, Collins in her article has recommended obtaining at least three pieces from two different sites in the esophagus including the distal and either mid or proximal esophagus[43]. If there is a high suspicion of the presence of EE, then biopsies should be obtained even if the esophagus endoscopically appears normal. Liacouras *et al*[60] found in their chart review that about one third of the patients with severe EE had a visually normal esophagus and they recommended that one should not rely only on the endoscopic appearance and rather aim to get biopsies for histological analysis if there is suspicion for EE.

Table 4 Diagnostic guidelines for eosinophilic esophagitis

Eosinophilic esophagitis	
Symptoms (adults)	GERD refractory to medical therapy
	Dysphagia
	Food Impaction
	Retrosternal chest pain
Endoscopy	Mucosal furrows
	Exudates
	Esophageal lumen narrowing
	Rings
Histology	Esophageal biopsies ≥15 eosinophils/HPF
	Biopsies obtained after 6-8 wk of *bid* PPI therapy or patients must have a documented negative pH study
	Normal biopsies in the rest of the GI tract

EE is thought to be primarily a TH2 inflammatory process[25,61] together with a possible allergic association, and as such, diagnosis also focuses on interleukins, eotaxin and eosinophils with their degranulation products. Research is ongoing regarding the development of non-invasive markers for EE. Gupta[62] reviewed some biomarkers that could correlate with disease presence, remission, severity and response to therapy. These include serum IgE, CD23, eotaxins, IL-5, MBP, eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN). Baxi *et al*[63] found the presence of peripheral blood eosinophilia in 67% of EE patients. However, many of these tests are not readily available, are expensive, time consuming and, as yet, have not been recommended for routine diagnosis of EE. More research thus needs to be done to correlate these tests with disease severity and patient demographics and to establish accurate and precise laboratory investigative methods and normal values[62] before they become part of the mainstream diagnostic tool scenario.

TREATMENT

Treatment modalities for patients with EE include pharmacological, endoscopic or dietary interventions used either singly or in combination. Endpoints of treatment are still not clear regarding whether relief of symptoms or esophageal inflammation need to be resolved. Clinicopathologically, EE involves esophageal eosinophilia and other causes of esophageal eosinophilia such as inflammatory bowel disease, parasitic infestation and GERD need to be ruled out[64].

Acid suppression

The association between GERD and EE is as yet unclear. Persistent reflux disease may cause esophageal eosinophilia, or EE may lead to secondary GERD[47] or simply, they may co-exist[31]. Acid suppression with PPIs helps to exclude GERD because EE is defined by a lack of response to PPI therapy[1]. There is some controversy as to this definition since Molina-Infante *et al*[65] showed that clinical response to PPIs does not completely rule

out quiescent EE. Furthermore, pathological diagnosis of EE should be done after a patient has been on PPI therapy for at least 4 wk. An important role of the use of PPI in patients with EE is symptomatic relief because of the multilayer involvement of the esophagus and the possibility of secondary GERD[64].

Systemic steroids

Systemic steroids are effective in managing EE. Liacouras *et al*[60] conducted a 10-year retrospective study of 381 patients diagnosed with EE and found that systemic corticosteroids significantly improved clinical symptoms and esophageal histology. Unfortunately EE recurred after withdrawal of steroids. Thus, side effects and recurrence after withdrawal limit their usage in management of EE.

Topical steroids

Arora *et al* evaluated 21 patients with dysphagia and treated them with swallowed fluticasone. Relief of dysphagia occurred in all and symptom relief lasted at least 4 mo. Schaefer *et al*[66] compared oral prednisone and swallowed fluticasone and found no clinical advantage of prednisone over fluticasone. Symptom relief and histological improvement were observed in both treatment groups. Symptom relapse was seen in both groups upon discontinuation of therapy, thus necessitating the need for long-term maintenance protocols. Because of the higher risk of systemic side effects and the need for maintenance therapy, topical therapy may actually turn out to be a better option. Currently, there are no steroids developed specifically for EE. Aceves *et al*[67] described a case series of two children who benefited from treatment with a viscous suspension of budesonide but not with fluticasone. However, studies to determine the efficacy of different topical steroids, methods of preparation and long-term maintenance need to be performed to recommend any one steroid over another.

Leukotriene inhibitors

Leukotrienes are eosinophil chemoattractants and hence one would expect that blocking leukotrienes may decrease eosinophilic migration and accumulation. Attwood *et al*[68] studied 12 patients who hitherto had been unresponsive to conventional therapy and started eight of them on montelukast. Six of these patients reported complete subjective improvement. Preceding this, there had been reports of an association between zafirlukast, another leukotriene inhibitor, and Churg-Strauss syndrome[69,70]. This association has not yet been seen with montelukast, but further studies are needed to determine the risks and benefits of using leukotriene inhibitors in patients with EE.

Biologics

IL-5 is a cytokine that plays a role in eosinophil regulation[71], and as such, inhibiting IL-5 could play a role in decreasing eosinophil-mediated inflammation. Garrett *et al*[72] performed an open label trial of mepolizumab, a humanized blocking monoclonal antibody against IL-5, in four patients with hypereosinophilic syndrome, and found it to be effective and safe with steroid-sparing properties. This was later corroborated by Stein *et al*[73] and anti-IL-5 seems to be a promising new therapy in patients with EE.

Immunomodulators

Netzer *et al*[74] evaluated three patients with corticosteroid-dependant EE and found that azathioprine and 6-mercaptopurine induced clinical and histological remission in all of them. More studies are indicated in this area, especially since treatment with immunomodulators can potentially help in decreasing the side effects associated with chronic steroid use.

Elemental and elimination diet

Infiltration of the esophagus with eosinophils forms the hallmark of EE. Because of the close association of EE with other allergic disorders, avoidance of presumed allergens provides a rationale for the use of an elimination diet in patients with EE. An elemental diet is one in which all solid foods are replaced with a nutritionally complete elemental formula and the protein source is comprised entirely of synthetic amino acids[75], whereas an elimination diet attempts to avoid including possible food allergens in a person's daily diet. Kelly *et al*[5] studied 10 children with GERD refractory to standard medications and fed them elemental diet followed by repeat endoscopy and food challenges. Symptomatic improvement with a decrease in eosinophils was seen in all patients. Symptoms relapsed after these patients were exposed to food challenges. This pioneering work formed the basis for many follow-up trials, which reported success with elimination diets. Markowitz *et al*[6] found that patients responded symptomatically and histologically to an elemental diet. Further confirmation of the success of an elemental diet was also confirmed using more formal evaluation with skin prick and atopy patch testing[76,77]. Sugnanam *et al*[78] then analyzed prospectively the sensitization profile of food and inhalant allergens in their cohort of patients with EE, by performing skin prick and patch testing. They found that younger patients showed more IgE and patch sensitization to food allergens. Spergel *et al*[77], in their retrospective study analyzing the relation between skin prick and atopy patch testing and food elimination diet in patients with EE, found that a large number of their patient population had normalization of biopsy results on elimination and reoccurrence on reintroduction. Kagalwalla *et al*[16] used a six-food elimination diet rather than the conventional elemental diet and found it to be associated with good clinical and histological response. The major problem with an elemental diet is the lack of palatability and thus a six-food elimination diet offers the advantage of better acceptability and compliance[16]. Elemental formulae do not contain fiber, and other nutrients may not be available based on the formula used in any particular patient. In these situations, fiber supplementation may be useful, especially in children or those who are prone to constipation, and other nutrients may be provided by other foods[75]. It is also beneficial

to have a registered dietician or a nutritionist involved because elimination diets may have a significant impact on the whole family who will need to be educated on the type of food that the patient can safely eat and on balancing the daily nutritional requirements of the individual. Food reintroduction forms an important aspect of management. Spergel and Shuker, in their article on nutritional management of EE, advocate reintroducing the least allergic foods followed by the most allergic ones. Periodic endoscopies are performed to assure symptomatic and histological improvement. If symptoms reappear, then that food is avoided, but by using this approach, patients can go back to an appropriate diet acceptable to the patient and the family[75].

Endoscopic dilatation

EE is characterized by eosinophilic infiltration that may extend into deeper layers of the esophagus[1] and by subsequent chronic inflammation causing tissue remodeling including subepithelial fibrosis[45]. Endoscopically, this may present as luminal narrowing, stricture formation or decreased tissue compliance[40], wherein, patients typically present with chronic dysphagia or foreign-body impaction. Food impaction is one of the commonest causes of dysphagia, and is considered an alarm symptom warranting immediate evaluation. The push technique has previously been advocated in acute esophageal food impaction[79,80]. Recent reports have suggested a prevalence of EE in at least 50% of patients with esophageal food impaction[81,82]. EE therefore is now increasingly being considered in patients presenting with the above symptoms. However, Kaplan *et al*[50] reported that tearing of the esophagus can occur even with routine passage of the endoscope, and because of this, dilatation was recommended after careful consideration only in those patients non-responsive to medical therapy and having rings obstructing the lumen. Fox in his article reported that longitudinal tearing or splitting of the mucosa is occasionally appreciated only during withdrawal of the endoscope[40], thus extreme care is warranted is selecting patients for endoscopic evaluation and dilatation. In a recent article, Straumann cautions against food bolus removal with rigid endoscopy in patients suspected of having EE, because of the high rate of perforation[52]. Other reports, however, suggest endoscopic dilatation to be a relatively safe procedure. Croese *et al*[8] found that 87% of their patients with EE had tears but none had serious complications, thus indicating dilation to be a safe intervention in patients with strictures. It will be worthwhile to conduct trials to evaluate whether the frequency of endoscopic dilations or the risk of complications with endoscopic maneuvers decrease if patients have prior medical treatment.

CONCLUSION

Eosinophilic esophagitis is increasingly being recognized in the adult population. It can present with a variety of symptoms including dysphagia and food impaction, along with other nasal and trachea-bronchial symptoms. Long-term sequelae of EE may include secondary malnutrition, weight loss, and acute esophageal perforations. Diagnosis of EE involves clinicopathological criteria and endoscopic biopsies. Because of the absence of a single known factor involved in the pathogenesis of EE, treatment options are multiple and include acid suppression, steroids, leukotriene inhibitors, elemental and elimination diets, and endoscopic dilations. Careful selection of patients must be done before the initiation of therapy because of the inherent risks and acceptability involved in each of them.

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Gastroparesis: Current diagnostic challenges and management considerations

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Abstract

Gastroparesis refers to abnormal gastric motility characterized by delayed gastric emptying in the absence of mechanical obstruction. The most common etiologies include diabetes, post-surgical and idiopathic. The most common symptoms are nausea, vomiting and epigastric pain. Gastroparesis is estimated to affect 4% of the population and symptomatology may range from little effect on daily activity to severe disability and frequent hospitalizations. The gold standard of diagnosis is solid meal gastric scintigraphy. Treatment is multimodal and includes dietary modification, prokinetic and anti-emetic medications, and surgical interventions. New advances in drug therapy, and gastric electrical stimulation techniques have been introduced and might provide new hope to patients with refractory gastroparesis. In this comprehensive review, we discuss gastroparesis with emphasis on the latest developments; from the perspective of the practicing clinician.

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Key words: Gastroparesis, Nausea; Vomiting; Prokinetic; Therapy

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INTRODUCTION

Gastroparesis is a condition of abnormal gastric motility characterized by delayed gastric emptying in the absence of mechanical outlet obstruction. The true prevalence of gastroparesis is unknown; however, it has been estimated that up to 4% of the adult population experiences symptomatic manifestations of this condition. A large study on long-term outcomes of gastroparetic adults revealed that 82% of patients were female[1]. Gastroparesis has a higher prevalence in the patient population of tertiary medical centers than in the community hospital setting. Moreover, a widely available diagnostic test that could be applied in a standard fashion is currently lacking in the primary care setting.

PATHOPHYSIOLOGY

Gastric motility results from the integration of tonic contractions of the fundus, phasic contractions of the antrum, and inhibitory forces of pyloric and duodenal contractions[2]. These contractions require a complex interaction between gastric smooth muscle, the enteric nervous system and specialized pacemaker cells, the interstitial cells of Cajal (ICC)[3]. Motor dysfunction of the stomach may result from autonomic neuropathy, enteric neuropathy, abnormalities of ICCs, fluctuations in blood glucose and psychosomatic factors[4-6].

The etiology of gastroparesis is multifactorial (Table 1). The three most common etiologies are diabetes, idiopathic, and post-surgical, especially if the vagus nerve is damaged. Other causes include medication, Parkinson's disease, collagen vascular disorders, thyroid dysfunction, liver disease, chronic renal insufficiency, intestinal pseudo-obstruction and

Table 1 Causes of gastroparesis

Causes	
Idiopathic	Medications: opiates, anticholinergics, β-adrenergics, Ca-channel blockers, glucagon, THC, alcohol, tobacco, *etc*
Surgical causes	Vagotomy and gastric resection/drainage Fundoplication, esophagectomy Gastric bypass surgery Whipple procedure Heart/lung transplant
Infections	Viruses-EBV, varicella, parvovirus-like Chagas disease *Clostridium Botulinum*
Central nervous system disorders	Cerebrovascular accidents/trauma Tumors Labyrinthine disorders Seizures
Peripheral nervous system disorders	Parkinson's disease Guillain-Barre Multiple sclerosis Dysautonomias
Neuropsychiatric disorders	Anorexia nervosa/bulimia Rumination syndrome
Rheumatologic disease	Scleroderma Systemic lupus erythematosus Polymyositis/dermatomyositis
Endocrine and metabolism diseases	Diabetes Hypothyroidism Parathyroid disease Electrolyte disorders Renal failure Pregnancy Neoplastic(para)-breast, small cell lung, pancreas
Misc. neuromuscular diseases	Amyloidosis Chronic intestinal pseudoobstruction Myotonic dystrophy

miscellaneous[1,7].

Originating in the region of ICCs, electrical activity in the form of gastric slow waves sweeps across the stomach toward the pylorus. However, these slow waves do not directly result in contraction of the gastric smooth muscle, but instead cause a simultaneous release of neurotransmitters from the enteric nerve endings, leading to smooth muscle contraction. Although neurohumoral control of gastric emptying is incompletely understood, both motilin and ghrelin are peptides secreted by the gastrointestinal endocrine cells that have been shown to increase gastric motor function[8,9].

In general, several factors affect gastric motility. These include motor dysfunction i.e. hypomotility and pyloric spasm, sensory dysfunction (such as impaired fundic relaxation, accommodation and abnormal sensation), electrical dysfunction (such as gastric arrhythmias and abnormal propagation), CNS effects resulting in nausea and vomiting, and others such as bacterial overgrowth, visceral hyperalgesia and gastrointestinal hormones.

SYMPTOMS AND EVALUATION

Gastroparesis is diagnosed by the presence of delayed gastric emptying in a symptomatic patient after other potential etiologies such as ulcer disease, mechanical obstruction, gastric cancer or other malignancies are excluded[10,11]. Symptoms of gastroparesis include nausea, vomiting, early satiety, bloating, post-prandial fullness, abdominal pain, weight loss and/or weight gain. These symptoms are non-specific and may mimic other disorders[10]. A simple severity grading scale has been proposed for stratification of symptoms[12] (Table 2). Also, a patient-based symptom instrument, the gastroparesis cardinal symptom index (GCSI) has been developed to assess severity of gastroparesis[13]. The GCSI total scores are based on three subscales of nausea/vomiting, post-prandial fullness/early satiety, and bloating. The GCSI scale is used to rate symptom change by either physicians or by the patient's own self-evaluations. In 146 patients with gastroparesis, nausea was present in 92%, vomiting in 84%, abdominal bloating in 75%, and early satiety in 60%. Abdominal pain or discomfort was present in 46%-89% of patients but was not the predominant symptom[1]. Abdominal pain in gastroparesis responds poorly to treatment[14]. Constipation may also be associated with gastroparesis. Treatment of constipation with an osmotic laxative has shown to improve dyspeptic symptoms as well as gastric emptying delay[15]. Complications of gastroparesis include esophagitis, Mallory-Weiss tear from chronic nausea/vomiting, malnutrition, volume depletion with acute renal failure (secondarily), electrolyte disturbances and bezoar formation[16,17].

Table 2 Proposed classification of gastroparesis severity

Classification	
Grade 1: Mild gastroparesis	Symptoms relatively easily controlled Able to maintain weight and nutrition on a regular diet or minor dietary modifications
Grade 2: Compensated gastroparesis	Moderate symptoms with partial control with pharmacological agents Able to maintain nutrition with dietary and lifestyle adjustments Rare hospital admissions
Grade 3: Severe gastroparesis	Refractory symptoms despite medical therapy Inability to maintain nutrition *via* oral route Frequent emergency room visits or hospitalizations

DIAGNOSTIC TESTS

Radiographic tests

Gastric scintigraphy: Gastric emptying scintigraphy of a radiolabeled solid meal is the gold standard for the diagnosis of gastroparesis. This test provides a physiological, non-invasive and quantitative measure of gastric emptying. Measurement of emptying of solids is more sensitive by scintigraphy. This is due to the fact that liquid emptying may remain normal despite advanced disease. A variety of foods including chicken, liver, eggs, egg whites, oatmeal, or pancakes are used as meals. The content of the meal is one of the most important

variables in gastric emptying. Solids versus liquids, indigestible residue, fat content, calories and volume of the test meal, can all alter gastric emptying time. Consensus recommendations for a standardized gastric emptying procedure have recommended a universally acceptable 99-m technetium sulfur-colloid labeled low fat, egg-white meal[18]. Medications that alter gastric emptying may be discontinued 48-72 h in advance, blood glucose in diabetics should be < 275 mg/dL on the day of the test and scinti-scanning at a minimum of 1, 2 and 4 h after test meal ingestion is performed in the upright position. This periodic measurement of radiolabeled solid meal has a specificity of 62% and a sensitivity of 93% when compared to continuous scinti-scanning[19]. Emptying of solids exhibits a lag phase followed by a prolonged linear emptying phase. The results of this test can be reported in two ways. The simplest approach is to report percent retention at defined times (minimum 1, 2, and 4 h). Half-times ($T_{½}$ values) may also be calculated but may potentially be less accurate, particularly in patients with very long emptying for whom extrapolation is needed to calculate the half-time if it was not actually reached during the test. Retention of over 10% of the solid meal after 4 h is abnormal. A grading of severity based on 4 h values might be used: grade 1 (mild), 11%-20% retention at 4 h; grade 2 (moderate), 21%-35% retention at 4 h; grade 3 (severe), 36%-50% retention at 4 h; and grade 4 (very severe), > 50% retention at 4 h[18]. Prokinetics may also be administered intravenously after the last measurement (i.e. 4 h) to evaluate if the patient is a "responder" or "non-responder" to the agent. Again, percent retained or extrapolated $T_{½}$ times can be calculated to assess the response. The drawbacks of the test include lack of standardization in different academic institutions, despite the current consensus recommendations, and radiation exposure, which is equivalent to about 1/3 of the average annual radiation exposure in the US from natural sources.

Radiopaque markers: After ingestion of indigestible markers, i.e. 10 small pieces of nasogastric tubing, none of the markers should remain in the stomach on an X-ray taken 6 h after ingestion with a meal[20]. This simple test correlates with clinical gastroparesis and is readily available and inexpensive. The drawbacks of the test include lack of standardization of the meal and size of markers and difficultly of determining if the markers are located in the stomach or other regions that overlap with the stomach (e.g. proximal small bowel, transverse colon).

Ultrasonography: Transabdominal ultrasound has been used to measure emptying of a liquid meal by serially evaluating cross-sectional changes in the volume remaining in the gastric antrum over time[21-23]. Emptying is considered complete when the antral area/volume returns to the fasting baseline. Some studies have revealed gastric emptying measurements similar to those seen with scintigraphy[24]. Three-dimensional ultrasound is a newly-developed technique that has recently been reported to be useful in determining stomach function[25,26] and duplex sonography can quantify transpyloric flow of liquid gastric contents. These techniques may be preferred over scintigraphy in patients such as pregnant women or children, in order to minimize radiation exposure. Drawbacks of the test include the fact that it is somewhat operator dependent, has proven reliable only for measurements of liquid emptying rates[24], and is less reliable when the patient is obese or when excessive gastric air is present. Moreover, liquid emptying is rarely impaired in patients with severe gastroparesis.

Magnetic resonance imaging: MRI using gadolinium has been found to accurately measure semi-solid gastric emptying and accommodation using sequential transaxial abdominal scans[27]. MRI provides excellent special resolution with a high sensitivity. It is also non-invasive and radiation free. Antral propagation waves can be observed and their velocity calculated. In gastroparesis, a significant reduction is seen in the velocity of these waves[28]. MRI can also differentiate gastric meal volume and total gastric volume, allowing gastric secretory rates to be calculated. New rapid techniques allow careful measurements of wall motion in both the proximal and distal stomach during emptying, and solid markers now permit measurement of solid meal emptying[29,30]. The drawback of this test is the expense and lack of availability.

Single-photon emission CT: This technique uses intravenously administered 99-Tc pertechnetate that accumulates within the gastric wall rather than the lumen and provides a three-dimensional outline of the stomach[31]. Measurement of regional gastric volumes in real-time to assess fundic accommodation and intragastric distribution can be made. The drawback of this test is the need of large radiation doses, and wide unavailability.

Stable isotope breath tests

The non-invasive 13-C-labeled octanoate breath test is an indirect means of measuring gastric emptying. It is a medium chain triglyceride which is bound to a solid meal such as a muffin. After ingestion and stomach emptying, 13-C octanoate is rapidly absorbed in the small intestine and metabolized to 13 CO_2 which is expelled from the lungs during expiration. The rate limiting step for the signal appearing in the breath is the rate of gastric emptying. Compared to detailed scintigraphy done over a period of 4 h, the breath test has a specificity of 80% and sensitivity of 86%[32]. The test assumes normal small bowel, pancreas, liver and pulmonary functions. Some studies have demonstrated a strong correlation between the carbon-labeled breath test and gastric scintigraphy[33,34]. The drawback of this test is the need for normal small intestinal absorption, liver metabolism, and pulmonary excretion to validate the test results.

Swallowed capsule telemetry

The ingestible "SmartPill®" (VA Boston Healthcare

System, MA, USA), or telemetry capsule, offers a promising new non-radioactive method for assessing gastric emptying. This capsule measures pH, pressure and temperature using miniaturized wireless sensor technology. This has been developed for ambulatory assessment of GI transit. The time taken for the pill to be expelled from the stomach into the duodenum is measured by monitoring the time point at which the acid readings of the stomach are replaced by the dramatic increase in pH as the capsule enters the duodenum. It has been shown that gastric transit time calculated using the SmartPill correlates well with gastric scintigraphy with good sensitivity (82%) and specificity (83%)[35]. The frequencies and amplitudes of antral contractions can be used to calculate motility indices. A current drawback is the cost of the pill and lack of widespread availability.

Antroduodenal manometry

In antroduodenal manometry, a water-perfused or solid-state manometric catheter is passed from the nares or mouth and placed fluoroscopically into the stomach and small bowel to measure actual gastroduodenal contractile activity. The frequency and amplitude of fasting, interdigestive and post-prandial contractions can be recorded, and the response to prokinetic agents can be assessed. Distinct patterns characterize the fasting and fed phases. During the fasting period, three cyclical phases known as migrating motor complex (MMC) recur approximately every 2 h: Phase Ⅰ, Phase Ⅱ and Phase Ⅲ. Phase Ⅰ is a period of motor quiescence followed by Phase Ⅱ, a period of intermittent phasic contractions. Phase Ⅲ, considered the "intestinal housekeeper", consists of an integrated peristaltic wave, initiated in the antrum, that sweeps indigestible solids from the stomach into the duodenum and beyond. Feeding disrupts the MMC and replaces it with a fed motor pattern of more regular antral contractions of variable amplitude that are either segmental or propulsive in character.

Gastroparesis is characterized by loss of normal fasting MMC's and reduced postprandial antral contractions and, in some cases pylorospasm[36]. Small intestinal motor dysfunction is detected in 17%-85% of patients with gastroparesis[37]. Manometry can also distinguish between myopathic and neuropathic small intestinal dysmotilities. However only in approximately 20%-25% of patients diagnosed with dysmotility syndromes by antroduodenal manometry, is clinical management influenced[38]. Antroduodenal manometry is usually reserved for the refractory gastroparesis patient evaluated at tertiary referral centers with the benefit of provocative testing to assess manometric response to treatment[39]. Drawbacks are that it is an invasive procedure, it needs motility expertise to perform and interpret the results, giving rise to problems with over interpretation in the unskilled hands.

Electrogastrography (EGG)

EGG measures gastric slow-wave myoelectrical activity *via* serosal, mucosal or cutaneous electrodes. It is most conveniently recorded with cutaneous electrodes positioned along the long axis of the stomach. Initially a pre-prandial recording for 45-60 min is captured. Patients are given a 500 kcal cheese or turkey sandwich and an equivalent postprandial recording is captured. The recorded signals are amplified and filtered to exclude contamination by noise from cardiorespiratory activity and patient movement. Computer analysis converts raw EGG signals to a three-dimensional plot. In healthy persons, EGG recordings exhibit uniform waveforms of 3 cycles/min, which increase in amplitude after ingestion of a meal. Abnormality of EGG is defined by rhythm disruption of more than 30% of the recording time including tachygastria (frequency of > 4 cycles/min) and bradygastria (< 2 cycles/min) and a lack of signal amplitude with eating[40]. EGG abnormalities are present in 75% of patients with gastroparesis[40]. EGG is considered by some authors as more of an adjunct to gastric emptying measurement for a comprehensive evaluation of patients with refractory symptoms[40]. Drawbacks are the little documented utility of EGG in the management of patients with suspected gastric dysmotility and movement artifacts that make recordings difficult to interpret.

Other tests

The gastric barostat test consists of a high compliance balloon device placed into the stomach to measure pressure-volume relationships and visceral sensation[41]. The drawback of this test is that it is invasive and is used therefore only as a research tool in a few tertiary centers.

The satiety test involves ingestion of water or a liquid nutrient until the patient reports maximal fullness. This test is not frequently performed and its main drawback is that results are subjective.

A common misconception is the use of barium upper gastrointestinal testing in the diagnosis of gastroparesis. Although this test can be used to evaluate anatomic abnormalities such as gastric outlet obstruction, it is not a functional study for the diagnosis of gastroparesis and other lesions such as malignancy may still be missed.

TREATMENT

The general principles of treatment of symptomatic gastroparesis are to: (1) correct fluid, electrolyte, and nutritional deficiencies; (2) identify and rectify the underlying cause of gastroparesis if possible; and (3) reduce symptoms[12,42].

In addition, patient education and explanation of the condition is an integral part of treatment. The disabling chronic symptoms of gastroparesis impact profoundly on the patient's sense of wellbeing, mental state, behavior and social life. Sensitive caring from the clinical team and professional counseling might be necessary to help the patient cope with the disability. Patients should be informed that a number of drugs might be tried in an attempt to discover the optimal therapeutic regimen and that the aim of treatment is to control rather than cure the disorder[43].

The patient's drug list should be reviewed to eliminate drugs that can cause gastric dysmotility. Management can be tailored to the severity of the gastroparesis. For grade 1 (mild) gastroparesis, dietary modifications should be tried. Low doses of antiemetic or prokinetic medications can be taken on an as-needed basis. Grade 2 (compensated) gastroparesis is treated by combination of antiemetic and prokinetic medications given at scheduled regular intervals. These agents relieve the more chronic symptoms of nausea, vomiting, early satiety and bloating. They frequently have no effect on abdominal pain. In grade 3 (severe) gastroparesis or gastric failure, more aggressive treatments including hospitalizations for i.v. hydration and medications, enteral or parenteral nutritional support and endoscopic or surgical therapy may be needed[12].

Dietary manipulation

Dietary recommendations rely on measures that promote gastric emptying or, at least theoretically, do not retard gastric emptying. At the outset, it is advisable to introduce an experienced dietician who can discuss and explore the patient's tolerance of solids, semi-solids and liquids, as well as dietary balance, meal size and timing of meals. Fats and fiber tend to retard emptying, thus their intake should be minimized. This should be stressed as many of these patients who often concomitantly also have constipation, have been told to take fiber supplementation for treatment of their constipation. Multiple small low fat meals about four or five times each day should be recommended. Carbonated liquids should be avoided to limit gastric distention. Patients are instructed to take fluids throughout the course of the meal and to sit or walk for 1-2 h after meals. If the above measures are ineffective, the patient may be advised to consume the bulk of their calories as liquid since liquid emptying is often preserved in patients with gastroparesis. Poor tolerance of a liquid diet is predictive of a future poor success[12].

Correction of glycemic control

Patients with diabetes should be counseled to achieve optimal glycemic control. Hyperglycemia itself delays gastric emptying, even in the absence of neuropathy or myopathy, which is likely to be mediated by reduced phasic antral contractility and induction of pyloric pressure waves. Hyperglycemia can inhibit the accelerating effects of prokinetic agents[44]. Measures more likely to be effective include more aggressive glucose monitoring, with frequent dosing of short acting insulin preparations to prevent post-prandial hyperglycemia. Prevention of wide fluctuations of hyperglycemia may be more important than maintenance of a given steady-state blood glucose level[45]. Improvement of glucose control increases antral contractility, corrects gastric dysrhythmias and accelerates emptying.

Pharmacological therapy

The pharmacotherapy of gastroparesis is stepwise, incremental and long term. The most commonly used drug classes include pro-motility and anti-emetic agents. There has been little in the way of randomized controlled investigations directly comparing the different agents. Consequently, a selection of drugs is used by trial and error.

Prokinetic agents: Prokinetic medications enhance the contractility of the GI tract, correct gastric dysrhythmias, and promote the movement of luminal contents in the antegrade direction. Prokinetics may improve predominantly symptoms of nausea, vomiting and bloating. They do not seem to relieve abdominal pain and early satiety associated with gastroparesis. They should be administered 30 min before meals to elicit maximal clinical effects. Bedtime doses are often added to facilitate nocturnal gastric emptying of indigestible solids. The response to treatment is usually judged clinically rather than with serial gastric emptying tests because symptom improvements correlate poorly with the acceleration of gastric emptying[46]. A meta-analysis assessing benefits of four different drugs in 514 patients in 36 clinical trials reported that the macrolide antibiotic erythromycin is the most potent stimulant of gastric emptying, while erythromycin and the dopamine receptor antagonist, domperidone, are best at reducing symptoms of gastroparesis[47]. Several factors must be considered when choosing a prokinetic drug for patients with gastroparesis, including efficacy, toxicity, regional availability and cost.

(I) Erythromycin. Erythromycin is a macrolide antibiotic that is also a motilin receptor agonist[48]. The intravenous form is the most potent stimulant of solid and liquid gastric emptying[49,50]. Motilin is a polypeptide hormone present in the distal stomach and duodenum that increases lower esophageal sphincter pressure and is responsible for initiating the MMC in the antrum of the stomach[51,52]. Erythromycin binds to motilin receptors and hence increases the amplitude of antral peristalsis, triggers premature MMC phase Ⅲ activity, and stimulates gastric emptying[53]. Interestingly, erythromycin has also been shown to accelerate emptying in post-vagotomy and antrectomy patients[54]. This may be due to its stimulatory effects on the fundus.

Erythromycin should be started at a low dose (200 mg per 5 mL) and is most rapidly absorbed when administered as a suspension[55]. However, tachyphylaxis develops in patients on chronic erythromycin therapy, due to down-regulation of motilin receptors which can develop as early as a few days of initiating therapy[53]. If tachyphylaxis develops, erythromycin can be discontinued for 2 wk and then restarted again. Intravenous erythromycin is used occasionally for inpatients with severe refractory gastroparesis[55]. Common side effects include skin rashes, nausea, cramping and abdominal pain. A large cohort reported that erythromycin increases the risk of sudden cardiac arrest by 2.01 times when compared to control population[56]. The risk for death was further increased in those patients who also were on CYP3A (cytochrome P-450 3A) inhibitors such as selected antipsychotics, cardiac antiarrythmics, antifungals, calcium antagonists,

antidepressants, and anti-emetics. Therefore, prior to initiating EES therapy for treatment of gastroparesis, all these factors need to be considered. Although this has not undergone formal testing, in our institution, a QTc of 450 ms in men and 460 ms in women has been used as the cut-off value over which EES is not administered due to risk of QT prolongation.

(Ⅱ) Metoclopramide. Metoclopramide is a substituted benzamide with several prokinetic actions, which include combined serotonin 5-hydroxytryptamine (HT) 4 receptor agonism, dopamine D2 receptor antagonism, and direct stimulation of gut smooth muscle contraction. The drug also has anti-emetic effects *via* brainstem D2 receptor antagonism, vagal and brainstem 5-HT3 receptor antagonism. The prokinetic properties of metoclopramide are limited to the proximal gut. Metoclopramide increases esophageal, fundic and antral contractile amplitudes, elevates lower esophageal sphincter pressure, and improves antropyloroduodenal coordination. Metoclopramide is administered orally in pill or liquid suspension form. Intravenous forms commonly are reserved for inpatients that cannot retain oral medications. Subcutaneous administration has also been reported to provide symptom control[57]. At least five controlled trails and four open label series have studied the efficacy of metoclopramide in gastroparesis[10]. In these nine trials, symptoms improved in seven studies, but improvement in gastric emptying was noted in only five. Patients may develop tolerance to the prokinetic action of metoclopramide over time; however, its antiemetic effects are sustained[58]. Metoclopramide is effective for the short-term treatment of gastroparesis for up to several weeks[59,60]. The long-term utility of metoclopramide has not been proven[61]. Side effects of metoclopramide occur in up to 30% of patients and result from antidopaminergic effects on the CNS. Acute dystonic reactions such as facial spasm, oculogyric crisis, trismus, and torticollis occur in 0.2%-6% of patients and are often observed in patients less than 30 years of age and within 48 h of initiating therapy[62]. Drowsiness, fatigue, and lassitude are reported by 10% of patients. Metoclopramide can worsen depression. Other side effects include restlessness, agitation, irritability, akathisia and hyperprolactinemic effects. Prolonged treatment with metoclopramide can produce extrapyramidal symptoms. These symptoms usually subside with 2-3 mo of discontinuation of the drug. Irreversible tardive dyskinesia is a catastrophic consequence that occurs in 1% to 10% of cases when metoclopramide is taken for more than 3 mo[62]. This condition is disabling and can develop without warning, therefore, it should be discussed in detail with the patients or their families with documentation of the discussion in their medical record. The current standard has been to sign an informed consent to document communicating the risks of metoclopramide.

(Ⅲ) Domperidone. Domperidone, a benzimidazole derivative, is a peripheral dopamine D2 receptor antagonist with benefits similar to those of metoclopramide. Domperidone does not cross the blood-brain barrier and consequently it has fewer central side effects. Brainstem structures regulating vomiting are outside the blood-brain barrier, therefore, domperidone has potent central anti-emetic action. At least five controlled trials and four open case series have assessed domperidone in patients with gastroparesis and diabetic gastropathy[63]. Symptoms improved in all studies, but accelerated gastric emptying was not uniformly observed. Domperidone may show tachphylaxis on repeated administration[64]. Adverse reactions to domperidone are commonly related to hyperprolectinemia due to the porous blood-brain barrier in the anterior pituitary[65]. These include menstrual irregularities, breast engorgement, and galactorrhea. An intravenous formulation of domperidone was removed in 1980 due to generation of cardiac arrhythmias[66]. Domperidone is not approved by the FDA for prescription in the United States, although it can be obtained in Canada, Mexico, New Zealand, Europe, and Japan. It is available in the US with approval of local institutional review boards, through an FDA investigational new drug application (IND) to patients with gastroparesis refractory to other therapies.

(Ⅳ) Tegaserod. This is a 5-HT4 receptor partial agonist used in the treatment of constipation predominant irritable bowel syndrome. In healthy volunteers, the drug stimulates small-intestinal motility and post-prandial antral and intestinal motility. Tegaserod has been shown to accelerate gastric emptying in some[67] but not all studies of healthy volunteers[68]. Tegaserod was completely withdrawn form the US market in April 2008 due to a reported increase in the risk of cardiovascular adverse effects.

(Ⅴ) Cisapride. Cisapride is a 5-HT4 receptor agonist with weak 5-HT3 antagonist properties that once was widely used for gastroparesis. This drug was withdrawn from the market in the United States in 2000 because of numerous reports of sudden death from cardiac arrhythmias[69]. Although the drug is still available overseas in numerous countries and obtainable from overseas websites, a recent consensus document did not recommend its use in gastroparesis[12].

(Ⅵ) Bethanechol. Bethanechol is an approved smooth muscle muscarinic agonist that increases lower esophageal sphincter pressure and evokes fundoantral contractions but does not induce propulsive contractions or accelerate gastric emptying[70]. Rarely, the drug may be used as an adjunct with other prokinetic medications in patients refractory to standard treatment with prokinetics and anti-emetic drugs. Prominent adverse effects include abdominal cramps, skin flushing, diaphoresis, lacrimation, salivation, nausea, vomiting, bronchoconstriction, urinary urgency, and miosis. Dangerous cardiovascular effects include abrupt decreases in blood pressure in hypertensive patients and atrial fibrillation in patients with hyperthyroidism.

(Ⅶ) Drugs in research. (1) Motilin receptor agonists. (a) Azithromycin is a macrolide antibiotic similar to erythromycin. It has been postulated that azithromycin

is also a motilin receptor agonist. In preliminary studies, intravenous administration of azithromycin improves antroduodenal contractions as measured by manometry[71]. However, there are no data available revealing an improvement in gastric emptying rates or patient symptoms after the administration of i.v. or oral azithromycin. The potential benefit of azithromycin is the longer half-life (68 h) as compared to erythromycin (1.5-2 h) and thus the less frequent dosing may help improve compliance with the medication (once a day *versus* four times a day). Furthermore, azithromycin is not metabolized, and elimination is largely in the feces, following excretion into the bile, with less than 10% excreted in the urine. Thus, it does not utilize the P-450 pathway in the liver and has less adverse effects due to drug interactions. It also appears that azithromycin has lower pro-arrhythmic potential compare with erythromycin but nevertheless cardiac adverse events have been reported[72-74]. From that prospective, it seems prudent to check the length of the QTc interval prior to initiating azithromycin therapy as well. (b) Mitemcinal is also a macrolide derived motilin receptor agonist with prokinetic properties. It does not have any antimicrobial actions. It produced symptom benefit in patients with diabetic gastropathy who had a body mass index of < 35 kg/m^2 and with hemoglobin A1C values $< 10\%$[75]. In addition, tachyphylaxis was not observed during the study period. (c) Atilmotin is another motilin receptor agonist, which, when given i.v., has been shown to accelerate gastric emptying of liquids and solids in healthy subjects[76]. It is not known whether atilmotin has significant effects on symptoms in patients with gastroparesis. (d) Ghrelin is a neurohumoral transmitter secreted by the stomach and is believed to play a physiological role as a stimulant of food intake and is also structurally related to motilin. Ghrelin has prokinetic properties, and has been shown to accelerate gastric emptying of a test meal in diabetic patients with slow gastric emptying[77], as well as improve gastric emptying and decreased meal-related symptoms in patients with idiopathic gastroparesis[78]. (2) Dopamine antagonists and serotonin agonists. (a) Itopride is a new D2 antagonist with anti-acetylcholinesterase effects. This drug showed prokinetic properties in animal models as well as promising effects in functional dyspepsia[79]. However, in healthy subjects, itopride had no effect on gastric emptying[80]. (b) Sulpiride is a dopamine blocker used for psychiatric disorders. Initial studies have shown that oral levosulpiride is superior to placebo[81], and may be as effective as cisapride in relieving nausea and vomiting in patients with gastroparesis[82,83]. Although this drug is not new, further studies are of interest to see whether it deserves a more established position for these gastrointestinal indications. (c) Mosapride is a 5-HT4 receptor agonist that accelerates gastric emptying in healthy volunteers and patients with diabetic gastroparesis[84]. In contrast to cisapride, mosapride has little effect on potassium-channel activity and seems to exhibit a significantly lower cardiac dysrhythmogenic potential[85]. (d) Renzapride is a combined 5-HT4 receptor agonist and 5-HT3-receptor antagonist. Future studies are needed to determine if renzapride exhibits efficacy in gastroparesis[12]. (3) Miscellaneous. (a) Physiostigmine and neostigmine are muscarinic receptor activators that stimulate gut motor activity by increasing acetylcholine levels. These drugs increase gastric contractions but have limited action in accelerate gastric emptying. However, pyridostigmine has been recently noted to reduce symptoms in a patient with gastroparesis secondary to underlying autoimmune disease[86]. (b) Nizatidine is a H2-receptor antagonist which exhibits anticholinesterase activity and stimulates gastric emptying but its efficacy in long-term treatment of gastroparesis is unknown[87]. (c) Cholecystokinin receptor antagonists such as loxiglumide and dexloxiglumide accelerate gastric emptying in some studies. The utility of such agents in gastroparesis remains to be determined[88]. (d) Sildenafil is a phosphodiesterase 5 inhibitor which has been shown to restore gastric emptying of liquids in an animal model of diabetes[89]. Sildenafil also reduced the dysrhythmias of the stomach induced experimentally by hyperglycemia in humans[90]. On the other hand, a thorough study of the effects of sildenafil on human gastric sensimotor functions showed that the drug significantly increases postprandial gastric volume and slows liquid (though not solid) emptying rate[91]. Sildenafil has also been found to inhibit interdigestive motor activity of the antrum and duodenum[92]. Clinical trials are clearly needed before this medication can be considered for the treatment of gastroparesis.

Anti-emetic medications: It is likely that a component of the clinical benefits observed with some of the available prokinetic drugs, such as metoclopramide and domperidone, stem from their anti-emetic actions on brain-stem nuclei. Nausea and vomiting are the most disabling symptom of gastroparesis and anti-emetic agents without stimulatory activity are often used alone or in concert with prokinetic drugs to treat gastroparesis. Antiemetic medications act on a broad range of distinct receptors subtypes in the peripheral and central nervous systems. Like prokinetics, the choice of antiemetic is empirical and it is reasonable to try the less expensive therapies initially.

(Ⅰ) Phenothiazines. These are the most commonly prescribed traditional antiemetics which include prochlorperazine and tiethyperazine. These drugs are both dopamine and cholinergic receptor antagonists acting on the area postrema (chemoreceptor trigger zone) in the brainstem. Prochlorperazine can be administered in the tablet form, liquid suspension, suppository and by injection. Side effects include sedation and extra-pyramidal effects such as drowsiness, dry mouth, constipation, skin rashes and Parkinsonian-like tardive dyskinesia.

(Ⅱ) Serotonin 5-HT3 receptor antagonist. These medications include ondansetron, granisetron, and dolasetron and are useful for prophylaxis of

chemotherapy induced nausea and vomiting, as well as symptoms occurring post operatively or during radiation therapy. These drugs may act on the chemoreceptor trigger zone as well as on peripheral afferent nerve fibers within the vagus nerve[42]. Ondansetron has no effect on gastric emptying in healthy volunteers and patients with gastroparesis and moreover can cause constipation[93,94]. This class of drugs maybe helpful when all other drugs have failed to provide symptom relief and are best given on an as-needed basis.

(Ⅲ) Anti-histamines. Antihistamines acting on H1 receptors exhibit central antiemetic effects[42]. Commonly prescribed antiemetics include diphenhydramine, dimenhydrinate and meclizine. These agents are most useful to treat symptoms related to motion sickness. The mechanism of action is poorly understood but is likely to involve both labyrinthine and chemoreceptor trigger zones. Side effects include drowsiness, dry mouth, blurred vision, difficulty urinating, constipation, palpitations, dizziness, insomnia and tremor.

(Ⅳ) Low-dose tricyclic antidepressants. Tricyclic antidepressants (TCAs) impair gastrointestinal motility through their anticholinergic activity but have been shown to relieve nausea, vomiting and pain in functional dyspepsia[95,96]. In a recent publication, 88% of diabetic patients with nausea and vomiting reported benefits with TCAs[97], of which one third had delayed gastric emptying, suggesting that these agents may have utility in gastroparesis. However, formal prospective trials of these antidepressants for the treatment of gastroparesis have not been performed, thus their use is still considered off-label. Side effects of low-dose TCAs are uncommon, excessive sedation and dry mouth occasionally limits use.

(Ⅴ) Other antiemetics. (1) Cannabinoids. Cannabinoid drugs such as dronabinol have been studied for improvement of gastrointestinal symptoms from chemotherapy and appear to have potency similar to standard antidopaminergics. Their benefit for gastroparesis has not been evaluated and they may also delay gastric emptying. (2) Benzodiazepines. These are useful for anticipatory nausea and vomiting before chemotherapy, but their efficacy in gastroparesis is unknown. These drugs maybe useful for their sedating effects in those patients with associated anxiety. (3) Neurokinin NK1-receptor antagonists. These are new antiemetics which treat both acute and delayed chemotherapy-induced nausea and vomiting[98,99], but their actions on gastric motor activity and symptoms in gastroparesis are uninvestigated. (4) Corticosteriods. Corticosteriods are employed as antiemetics in the postoperative setting or in the prevention of chemotherapy-induced emesis. One individual with idiopathic myenteric ganglionitis exhibited improvement with corticosteroid therapy, confirming the inflammatory basis of some cases of upper gut dysmotility[100].

Complementary and alternative therapies: Ginger, a traditional Chinese antiemetic agent, has weak 5-HT3 receptor antagonist properties and has gastric slow wave antidysrhythmic effects in humans[101,102]. These therapies are often given for treatment of nausea and vomiting of diverse etiologies. Acupressure and electrical acustimulation on the P6 acupuncture point reduce nausea postoperatively, after chemotherapy, and during nausea of pregnancy. One group observed benefits with acupuncture in 35 diabetic gastroparesis patients[103].

Medications for control of symptoms other than nausea and vomiting: (1) Early satiety. Early satiety has been related to defects in fundic accommodation in patients with functional dyspepsia[104]. Nitrates, buspirone, sumitriptan, and selective serotonin reuptake inhibitors promote fundic relaxation in this condition[105,106]. The use of fundic relaxants in managing early satiety in gastroparesis has not been investigated; (2) Abdominal pain. Epigastric pain is disabling in some individuals with gastroparesis and can result in excessive utilization of healthcare resources. The pathogenesis of pain is poorly understood and treatments for this symptom are largely unsatisfactory. Pain in gastroparesis has been postulated to be due to sensory rather than motor dysfunction, and therapies to reduce afferent dysfunction may be more effective for this symptom[107]. However, this hypothesis has not been tested. Although, non-steroidal anti-inflammatory drugs (NSAID's) have been shown to ameliorate gastric slow wave dysrhythmias in several healthy subjects[108], their adverse effects including renal dysfunction and ulcerogenic properties, limit their usage on a chronic basis. Antidepressant medications may help with gastroparesis associated neuropathic pain[109]. These include low dose tricyclic antidepressants (TCA), selective serotonin reuptake inhibitors (SSRIs), selective noradrenaline reuptake inhibitors (SNRIs) and combined serotonin/noradrenaline reuptake inhibitors. Paroxetine, an SSRI, may selectively accelerate small intestinal transit[110,111]. Opiates, including milder agents such as tramadol, should be avoided because of their inhibitory effects on motility as well as risk of addiction. (3) Nutritional support, enteral and parenteral. Some patients with refractory gastroparesis benefit from enteral or parenteral nutrition intermittently for symptom flares or for permanent support. Patients with chronic symptoms of gastroparesis may develop dehydration, electrolyte abnormalities and/or extreme malnutrition. The choice of nutritional support and its administration route depends on the severity of disease. The indications for supplementation of enteral nutrition include unintentional loss of 10% or more of the usual body weight during a period of 3 to 6 mo, inability to achieve the recommended weight by the oral route, repeated hospitalizations for refractory symptoms, interference with delivery of nutrients and medications, need for nasogastric intubation to relieve symptoms, and nausea and vomiting resulting in poor quality of life[12].

Except in cases of profound malnutrition or electrolyte disturbance, enteral feeding are preferable to chronic parenteral nutrition because of the significant risks of infection and liver disease in the latter treatment. On the other hand, short-term total parenteral nutrition (TPN) can reverse rapid weight decline and ensure adequate fluid delivery. Home intravenous TPN may be needed for individuals who cannot tolerate enteral feeding. Several options for enteral access and feeding are available and no data exists favoring one approach over the other. However, nasogastric tubes and gastrostomy tubes are not encouraged due to the possibility of worsening gastroparesis and risk of pulmonary aspiration. Jejunostomy tubes are preferred in order to bypass the gastroparetic stomach except if the patient has small bowel dysmotility. Short-term nasojejunal feeding is often used to help determine if the patient will tolerate chronic small bowel feeding through a permanent enteral access. Formulas that are low in osmolarity (e.g. Peptamen, Isocal) and with a caloric density of 1.0-1.5 cal/mL are recommended. A dietician should be consulted early on. Initially, infusion rates should be low and then advanced every 4-12 h as tolerated to meet caloric needs. Eventually, infusions can be converted to nocturnal feedings to free up daytime h for optional oral intake and to participate in normal daily activities.

Endoscopic treatment

Therapeutic endoscopy with pyloric injection of botulinum toxin A may provide benefit in some patient with gastroparesis. Botulinum toxin A is a bacterial toxin that inhibits acetylcholine release, causing muscle paralysis. Manometric studies in patients with diabetic gastroparesis have shown evidence of prolonged pylorospasm producing a functional outlet obstruction[36]. Several uncontrolled case series have reported reduced symptoms and acceleration of gastric emptying after botulinum toxin treatment[112-114]. The largest series reported 63 highly selected patients with primary idiopathic gastroparesis, 43% of whom responded symptomatically with mean response duration of 5 mo[115]. A double-blind controlled trial found no efficacy of botulinum toxin over placebo[116]. However, this report was underpowered to detect the effect of the drug. Another recent double-blind placebo-controlled trial revealed that intrapyloric injection of botulinum toxin improved gastric emptying in patients with gastroparesis, although this benefit was not superior to placebo at one month. Also, in comparison to placebo, symptoms did not improve significantly after 1 mo of injection[117]. The use of botulinum toxin for gastroparesis is considered off-label and should be considered when other accepted therapies have failed or produced unacceptable side effects. To date, few adverse effects have been reported with botulinum toxin therapy.

Surgical treatment

Surgical intervention is increasingly used to treat medically refractory/severe gastroparesis. Limited data are available concerning surgical treatment of gastroparesis[118]. The most common procedure is gastric electrical stimulation (GES). Other procedures offered include venting/feeding gastrostomy and jejunostomy tubes, surgical pyloroplasy, gastrectomy and surgical drainage procedures and pancreatic transplantation in diabetic patients. Apart from GES and feeding tubes, other surgical procedures are performed as a last resort in carefully evaluated patients with profound gastric stasis.

GES: Over the past decade, GES has been used for treatment of medically refractory gastroparesis[10,12,119]. Paced GES using an implantable stimulator (Enterra therapy, by Medtronic Inc.) has been approved by the FDA through a humanitarian device exemption. Electrical stimulation is delivered by two electrodes usually placed laproscopically on to the serosal surface of the stomach overlying the pacemaker area in the body of the stomach. Leads from the electrodes connect to a pulse generator that resembles a cardiac pacemaker that is implanted in a subcutaneous pocket of the anterior abdominal wall. The pulse generator delivers low energy 0.1-s trains of pulses at a frequency of 12 cycles/min. Within each pulse train, individual pulses oscillate at a frequency of 14 cycles/s[12]. Although the exact mechanism of action of the GES is unknown, the clinical effect is believed to be mediated by local neurostimulation. The stimulation impulses used are able to excite nerves but are too weak to excite gastric smooth muscles. Furthermore, poor correlation is observed between patients' symptoms and gastric emptying rates[119,120]. It has been hypothesized that the mechanism may stem from a vagal and cerebral pathway[121]; however, GES has been shown to work well even in patients with vagotomy[122]. Multiple uncontrolled studies in diabetic, idiopathic and post-surgical gastroparesis have shown efficacy of GES. In one uncontrolled multicenter trial, 35 of 38 patients experienced > 80% reductions in nausea and vomiting which persisted for 2.9-15.6 mo, with an associated 5.5% increase in weight and reduced requirement of supplemental nutrition[123]. Other studies reported similar long-term symptom benefits, which may persist for at least 10 years with improvements in body mass index, serum albumin and glycemic control[124,125]. In the only controlled trial of GES, 33 patients with idiopathic or diabetic gastroparesis completed a 2-mo double-blind, crossover, sham stimulation-controlled phase followed by 12 mo uncontrolled observation, with the device activated[119]. During the blinded phase, frequency of weekly vomiting in all patients was 6.8 times when the device was ON as opposed to 13.5 times when it was OFF. Although there was not a significant reduction in the total symptom score (TSS) in the ON *vs* OFF state, 21 patients preferred the stimulation ON, whereas seven preferred OFF and five had no preference. Symptom reductions were more impressive during the unblinded phase where median vomiting frequency decreased by > 80% for 50% of all patients. TSS was also significantly

improved in all patients from a score of 16.8 at baseline to 11.1 and 11.4 at 6 and 12 mo, respectively. The major adverse effect of GES is infection resulting in removal of the device in approximately 10% of patients[119,123]. The frequency of such infections seems to be decreasing during recent years. This may be explained by more careful surgical technique and the increasing use of laparoscopy instead of open surgery. The second concern is of the non-responder issue. In the earlier mentioned randomized trial[119] 13% of the patients were non-responders with < 25% symptom reduction. There seems to be a higher non-responder rate in idiopathic gastroparesis[125,126]. Abell and colleagues have applied temporary mucosal GES with endoscopically placed electrodes and used the effects on symptoms after ⩾ 3 d as a measure of response[127].

Other surgical options: In refractory patients with severe nausea and vomiting, placement of a gastrostomy tube for intermittent decompression by venting or suctioning may provide symptom relief, especially of interdigestive fullness and bloating secondary to retained intragastric gas and liquids. Pyloroplasty may be considered as another option but limited data are available on the efficacy of this procedure. There are limited controlled data concerning gastrectomy in gastroparesis[118]. A study of patients with near-total gastrectomy revealed long-term symptom relief in 43% patients with postsurgical gastroparesis[128]. The literature is sparse concerning correction of diabetic gastroparesis status post-pancreas and pancreas-kidney transplant in patients with type 1 diabetes[129,130].

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GUIDELINES CLINICAL PRACTICE

Dr. Peter V Draganov, *Series Editor*

Pancreatic pseudocyst

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Abstract

Pancreatic pseudocysts are complications of acute or chronic pancreatitis. Initial diagnosis is accomplished most often by cross-sectional imaging. Endoscopic ultrasound with fine needle aspiration has become the preferred test to help distinguish pseudocyst from other cystic lesions of the pancreas. Most pseudocysts resolve spontaneously with supportive care. The size of the pseudocyst and the length of time the cyst has been present are poor predictors for the potential of pseudocyst resolution or complications, but in general, larger cysts are more likely to be symptomatic or cause complications. The main two indications for some type of invasive drainage procedure are persistent patient symptoms or the presence of complications (infection, gastric outlet or biliary obstruction, bleeding). Three different strategies for pancreatic pseudocysts drainage are available: endoscopic (transpapillary or transmural) drainage, percutaneous catheter drainage, or open surgery. To date, no prospective controlled studies have compared directly these approaches. As a result, the management varies based on local expertise, but in general, endoscopic drainage is becoming the preferred approach because it is less invasive than surgery, avoids the need for external drain, and has a high long-term success rate. A tailored therapeutic approach taking into consideration patient preferences and involving multidisciplinary team of therapeutic endoscopist, interventional radiologist and pancreatic surgeon should be considered in all cases.

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Key words: Pancreatic pseudocyst; Cyst; Pancreatititis; Endoscopic ultrasound; Therapy

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INTRODUCTION

Pseudocyst of the pancreas is a localized fluid collection that is rich in amylase and other pancreatic enzymes and is surrounded by a wall of fibrous tissue that is not lined by epithelium[1]. Pseudocysts are connected with the pancreatic duct system, either as a direct communication or indirectly *via* the pancreatic parenchyma. They are caused by pancreatic ductal disruption following increased pancreatic ductal pressure, either due to stenosis, calculi or protein plugs obstructing the main pancreatic ductal system, or as a result of pancreatic necrosis following an attack of acute pancreatitis[2,3]. Pseudocysts are a common clinical problem and complicate the course of chronic pancreatitis in 30% to 40% of patients[4].

ETIOLOGY

The occurrence of pseudocyst parallels that of pancreatitis and the etiology of pseudocysts resembles the causes of pancreatitis closely, although pseudocyst formation is less common after acute compared to chronic pancreatitis, and it is more common after alcohol-induced than after non-alcohol-related pancreatitis. Alcohol-related pancreatitis appears to be the major cause in studies from countries where alcohol consumption is high and accounts for 59%-78% of all pseudocysts[5].

Walt *et al*[6] reported data collected from Wayne State University Hospital in Detroit, USA. The causative factors in the 357 admissions for pancreatic pseudocysts included alcohol use in 251 cases (70%), biliary tract disease in 28 (8%), blunt trauma in 17 (5%), penetrating trauma in four (1%), operative trauma in one (0.3%),

and idiopathic in 56 (16%). Most of the patients in the idiopathic group were thought to have been alcohol-related, but no definite evidence was recorded[6].

CLASSIFICATION

D'Egidio and Schein, in 1991, described a classification of pancreatic pseudocyst based on the underlying etiology of pancreatitis (acute or chronic), the pancreatic ductal anatomy, and the presence of communication between the cyst and the pancreatic duct[7]. They define three distinct types of pseudocysts[7]. Type Ⅰ, or acute "post-necrotic" pseudocysts, that occur after an episode of acute pancreatitis and are associated with normal duct anatomy, and rarely communicate with the pancreatic duct. Type Ⅱ, also post-necrotic pseudocysts, which occurs after an episode of acute-on-chronic pancreatitis (the pancreatic duct is diseased, but not strictured, and there is often a duct-pseudocyst communication). Type Ⅲ, defined as "retention" pseudocysts, occur with chronic pancreatitis and are uniformly associated with duct stricture and pseudocystduct communication.

Another classification, based entirely on pancreatic duct anatomy, is proposed by Nealon and Walser[8]. Type Ⅰ: normal duct/no communication with the cyst. Type Ⅱ: normal duct with duct-cyst communication. Type Ⅲ: otherwise normal duct with stricture and no duct-cyst communication. Type Ⅳ: otherwise normal duct with stricture and duct-cyst communication. Type Ⅴ: otherwise normal duct with complete cut-off. Type Ⅵ: chronic pancreatitis, no duct-cyst communication. Type Ⅶ: chronic pancreatitis with duct-cyst communication[8].

INCIDENCE

Regardless of the etiology of pseudocyst, the incidence is low, 1.6%-4.5%, or 0.5-1 per 100 000 adults per year[9,10]. In a study by Imrie, pseudocysts developed after emergency hospital admission for an episode of acute pancreatitis in 86 patients[11]. Sixty-two of the 86 pseudocysts consequent to acute pancreatitis were derived from the local hospital population area, in which 879 patients with acute pancreatitis were admitted to hospital during the same time period. This resulted in a 7% overall incidence of pseudocysts as a complication of acute pancreatitis[11].

In a series of 926 patients with non-alcoholic acute pancreatitis, fluid collections were observed in 83 (9%). At the end of 6 wk, 48 (5%) still had a fluid collection consistent with a pseudocyst[12].

Kourtesis *et al*[13] followed prospectively with computed tomography (CT) 128 consecutive patients with acute pancreatitis (mostly alcohol-induced). Forty-eight patients (37%) developed fluid collection in the pancreatic region. The majority of these resolved spontaneously. In 15 (12%) patients, symptomatic pseudocysts developed.

Pseudocysts tend to be more common in chronic as compared to acute pancreatitis. Incidence figures of 30% to 40% have been reported in the literature[4]. However, there is a lack of precise data based on the long-term follow-up of patients with chronic pancreatitis, in contrast to acute pseudocysts where the patient with chronic pancreatitis may have had the disease for 10, 20 or more years giving him a high risk of developing a pseudocyst at least once over a long period of sickness[14].

Table 1 Differential diagnosis of pancreatic pseudocyst

Pancreatic diseases	Extrapancreatic diseases
Acute & chronic pancreatitis	Peptic ulcer disease & gastric cancer
Pancreatic necrosis & abscess	Acute cholecystitis & gallstones
Adenocarcinoma of the pancreas	Abdominal aortic aneurysm
Pancreatic cystic neoplasms	Intestinal ischemia
Pancreatic artery pseudoaneurysm	Ovarian cysts & cancers
	Bowel obstruction
	Acute myocardial infarction
	Pneumonia

PATHOGENESIS

The pathogenesis of pseudocysts seems to stem from disruptions of the pancreatic duct due to pancreatitis or trauma followed by extravasation of pancreatic secretions. Two thirds of patients with pseudocysts have demonstrable connections between the cyst and the pancreatic duct. In the other third, an inflammatory reaction most likely sealed the connection so that it is not demonstrable.

In case of pseudocyst following an episode of acute pancreatitis, only if the acute fluid collection persists more than 4-6 wk, and is well-defined by a wall of fibrous or granulation tissue, can one say that an acute pseudocyst has appeared. Such a pseudocyst usually contains enzymatic fluid and necrotic debris[1,5].

The pathogenesis of pseudocyst formation in chronic pancreatitis is less well understood but, at least two mechanisms may be involved, the cyst may develop as a consequence of an acute exacerbation of the underlying disease and/or blockage of a major branch of the pancreatic duct by a protein plug, calculus or localized fibrosis[15].

CLINICAL PRESENTATION, DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

The clinical presentation of pancreatic pseudocyst can range from asymptomatic patient to major abdominal catastrophe due to complications[16-18]. Acute complications include bleeding (usually from splenic artery pseudoaneurysm), infection, and rupture.

Chronic complications include gastric outlet obstruction, biliary obstruction and thrombosis of the splenic or portal vein with development of gastric varices[18].

A variety of diseases can mimic the clinical presentation of pancreatic pseudocyst (Table 1). Once pancreatic cyst is identified by an imaging modality, the most important question is to differentiate pseudocyst from other cystic lesions of the pancreas (Table 2).

Table 2 Differential diagnosis of cystic pancreatic lesions

	SCA	MCN	IPMN	SPN	Pseudocyst
Prevalent age	Middle age	Middle age	Elderly	Young	Variable
Sex	Mostly female	Mostly female	Male > female	Mostly female	Male > female
Presentation	Mass/pain	Mass/pain	Pancreatitis	Mass/pain	Pain
Location	Evenly	Body/tail	Head	Evenly	Evenly
Malignant potential	Very low	Moderate to high	Low to high	Low	None

SCA: Serous cystadenoma; MCN: Mucinous cystic neoplasm; IPMN: Intraductal papillary mucinous neoplasia; SPN: Solid pseudopapillary neoplasm.

HISTORY AND PHYSICAL EXAMINATION

No specific set of symptoms is specific for pseudocysts; however, one should consider the possibility of a pseudocyst in a patient who has persistent abdominal pain, anorexia, or abdominal mass after a case of pancreatitis. Rarely, patients present with jaundice or sepsis from an infected pseudocyst[16]. Occasionally, even patients with large pancreatic pseudocyst are asymptomatic. In patients presenting with pancreatic cyst incidentally discovered on imaging, a crucial point is to define whether the patient has had prior history of pancreatitis. The sensitivity of physical examination findings is limited. Patients frequently have a tender abdomen. They can occasionally have a palpable abdominal mass. Peritoneal signs suggest rupture of the cyst or infection. Other possible findings include fever, scleral icterus or pleural effusion[17].

LABORATORY EVALUATIONS

Serum tests have limited utility. Amylase and lipase levels are often elevated, but may be within reference ranges. The serum bilirubin and liver chemistries may be elevated if the bile duct is obstructed from stone, extrinsic compression from the pseudocyst or from underlying liver disorder (e.g. alcoholic hepatitis). Some laboratory tests may provide clues to the underlying etiology of pancreatitis (e.g. elevated triglycerides or calcium level). Elevated liver chemistries raise the suspicion for biliary pancreatitis.

IMAGING MODALITIES

Transabdominal ultrasound (US)

Pancreatic pseudocyst appears as an echoic structure associated with distal acoustic enhancement on US examination. They are well defined and round or oval, and they are contained within a smooth wall. During the early phases of their development, pseudocysts can appear more complex, with varying degrees of internal echoes. Usually, this appearance results from the presence of necrotic debris and is more common in pseudocysts that form as a result of acute necrotizing pancreatitis than in chronic pancreatitis related pseudocysts. The debris is cleared over time in most cases. The pseudocyst can appear more complex in two other instances: when hemorrhage occurs into the cyst or when infection of the cyst complicates the clinical course. Color Doppler or duplex scanning should always be performed in cystic lesions to ensure that the lesion in question is not a giant pseudoaneurysm. Sensitivity rates for US in the detection of pancreatic pseudocysts are 75% to 90%. Therefore, US is inferior to CT, which has a sensitivity of 90% to 100%. US has several limitations, as compared with CT, in the initial diagnosis of a pseudocyst: the presence of overlying bowel gas decreases the sensitivity of US, and unlike CT, US examinations are highly operator dependent[19].

CT

The identification of a thick-walled, rounded, fluid-filled mass adjacent to the pancreas on an abdominal CT scan in a patient with a history of acute or chronic pancreatitis is virtually pathognomonic for pancreatic pseudocyst. Positive CT findings in this clinical situation do not require confirmation with another diagnostic modality. In the acute setting, a CT scan is the better choice because significant amounts of bowel gas resulting from ileus or obstruction decrease the sensitivity of US. In addition, CT scans provide more detailed information regarding the surrounding anatomy and can demonstrate additional pathology, including pancreatic duct dilatation and calcifications, common bile duct dilatation, and extension of the pseudocyst outside the lesser sac. The major weakness of CT scanning is the relative inability to differentiate pseudocyst from cystic neoplasm, especially mucinous cystadenomas and intraductal papillary mucinous neoplasm (IPMN)[20]. Furthermore, the intravenous contrast administered at the time of CT can precipitate or worsen kidney dysfunction.

Magnetic resonance imaging (MRI)

MRI and magnetic resonance cholangiopancreatography (MRCP) are sensitive diagnostic modalities for pancreatic pseudocysts. They are generally not routinely used because CT scanning typically offers all the diagnostic information that is required. However, the increased contrast provides for better characterization of fluid collections. MRI or MRCP is superior to CT in depicting debris within fluid collections and pseudocysts. On T2-weighted images, a fluid-filled cystic mass produces high signal intensity and appears bright. The pancreatic duct and biliary systems are easily visualized in detail, although interpreting the status of pancreatic duct integrity may be difficult[21].

The ability of MRI/MRCP to depict choledocholithiasis

Table 3 Cystic fluid analysis in cystic pancreatic diseases

	SCA	MCN	MCAC	Pseudocyst
CEA	Low	High	High	Low
CA125	Variable	Variable	High	Low
CA19-9	Variable	Variable-high	Variable-high	Variable
Amylase	Low-high	Low-high	Low-high	High
Lipase	Low	Low	Low	High

SCA: Serous cystadenoma; MCN: Mucinous cystic neoplasm; MCAC: Mucinous cystadenocarcinoma

is far superior to that of CT or US. Furthermore, MRCP techniques can also depict subtle branch-chain dilatation in chronic pancreatitis. MRI is also highly sensitive to detect bleeding with complex fluid collections.

Endoscopic retrograde cholangiopancreatography (ERCP)

ERCP is not necessary in diagnosing pseudocysts, but can provide definitive therapy in some cases. It also can be useful in planning possible drainage strategy. A study by Nealon *et al*[22] investigated the use of ERCP and the treatment of pseudocysts and acute pancreatitis and reported that ERCP findings may influence the treatment plan. Some authors, therefore, recommend performing an ERCP before contemplated surgical procedures. We believe that with the advent of alternative imaging technology [(CT, MRI, MRCP and endoscopic ultrasound (EUS)] ERCP is not necessary in most cases, but this has not been formally tested in a prospective study.

EUS

EUS is usually used as a secondary test to further evaluate pancreatic cyst detected by other imaging modality (US, CT or MRI). EUS is the test of choice when attempting to distinguish pancreatic pseudocyst from other cystic lesions of the pancreas. Visualization of the pancreas *via* EUS provides high quality images due to the close proximity of the ultrasound transducer to the area of interest. Criteria suggestive of cystic neoplasm include a cyst wall thickness of greater than 3 mm, macroseptation (all cystic components more than 10 mm), the presence of a mass or nodule, and cystic dilation of the main pancreatic duct[23-25]. Fine needle aspiration (FNA) of the cyst can be performed at the time of EUS and cyst fluid obtained for laboratory evaluation (see laboratory evaluation above). EUS can also be used to guide therapeutic endoscopic drainage.

Analysis of the cyst fluid may help differentiate pseudocysts from cystic tumors of the pancreas (Table 3). The preferred modality to obtain cystic fluid for analysis is EUS. Carcinoembryonic antigen (CEA) level in the cystic fluid is the marker most commonly used. It is low in pseudocysts and serous cystadenomas and elevated in mucinous cystadenomas. A CEA level of greater than 400 ng/mL within the cyst fluid strongly suggests mucinous lesion[23,24,26]. Amylase levels are usually high in pseudocysts and low in serous cystadenoma. Cytology is occasionally helpful, but a negative result does not exclude malignancy.

Hammel *et al*[27] published a study to assess the reliability of preoperative biochemical and tumor marker analysis in cyst fluids obtained by FNA for pathological diagnosis. Cyst fluid was obtained preoperatively by FNA, and biochemical and tumoral marker values were measured. The diagnosis of cystic tumors (seven serous cystadenomas and 12 mucinous tumors) was established by surgical specimen analysis. Thirty-one pancreatic pseudocysts complicating well-documented chronic pancreatitis were also studied. The results showed that carbohydrate antigen 19-9 levels of > 50 000 U/mL had a 75% sensitivity and a 90% specificity for distinguishing mucinous tumors from other cystic lesions. CEA levels of < 5 ng/mL had a 100% sensitivity and an 86% specificity for distinguishing serous cystadenomas from other cystic lesions. Amylase levels of > 5000 U/mL had a 94% sensitivity and a 74% specificity for distinguishing pseudocysts from other cystic lesions. His conclusion was: high carbohydrate antigen 19-9, low CEA, and high amylase levels in cyst fluid are very indicative of mucinous tumors, serous cystadenomas, and pseudocysts, respectively[27].

Sperti *et al*[28] published a study that was performed to evaluate the utility of serum and cyst fluid analysis for enzymes (amylase and lipase) and tumor markers (CEA, CA 19-9, CA 125, and CA 72-4) in the differential diagnosis of cystic pancreatic lesions. In the study, serum and cyst fluid were obtained from 48 patients with pancreatic cysts (21 pseudocysts, 14 mucinous cystic neoplasms, six ductal carcinomas, and seven serous cystadenomas), observed between 1989 and 1994. The results showed that serum CA 19-9 levels were significantly higher in ductal carcinomas (all > 100 U/mL) and mucinous cystic neoplasms ($P < 0.05$). CA 72-4 cyst fluid levels were significantly higher in mucinous cystic tumors ($P < 0.005$), with 95% specificity and 80% sensitivity in detecting mucinous or malignant cysts. A combined assay of serum CA 19-9 and cyst fluid CA 72-4 correctly identified 19 of 20 (pre-) malignant lesions (95%), with only one false-positive result (3.6%). Cytology showed a sensitivity of 48% and specificity of 100%. Their conclusion was that any pancreatic cyst with high serum CA 19-9 values, positive cytology, or high CA 72-4 in the fluid should be considered for resection[28].

Khalid *et al*[29] published a prospective study of the utility of molecular analysis of the pancreatic pseudocyst. In the study, endoscopic ultrasound-guided pancreatic cyst aspirates were prospectively collected during a period of 19 mo and studied for cytology, CEA level, and molecular analysis. Molecular evaluation incorporated DNA quantification (amount and quality), *κ-ras* point mutation, and broad panel tumor suppressor linked microsatellite marker allelic loss analysis by using fluorescent capillary electrophoresis. The sequence of mutation acquisition was also calculated on the basis of a clonal expansion model, and comparison was made to the final pathology. Thirty-six cysts with confirmed histology were analyzed. There were 11 malignant, 15

premalignant, and 10 benign cysts. Malignant cysts could be differentiated from premalignant cysts on the basis of fluid CEA level ($P = 0.034$), DNA quality ($P = 0.009$), number of mutations ($P = 0.002$), and on the sequence of mutations acquired ($P < 0.001$). Early *κ-ras* mutation followed by allelic loss was the most predictive of a malignant cyst (sensitivity, 91%; specificity, 93%). The study concluded that malignant cyst fluid contains adequate DNA to allow mutational analysis. A first hit *κ-ras* mutation followed by allelic loss is most predictive of the presence of malignancy in a pancreatic cyst. This approach should serve as an ancillary tool to the conventional work-up of pancreatic cysts. Cumulative amount and timing of detectable mutational damage can assist in diagnosis and clinical management[29].

TREATMENT OF PANCREATIC PSEUDOCYST

Supportive medical care

Intravenous fluids, analgesics and antiemetics are routinely given. For patients that can tolerate oral intake, low fat diet is recommended. In patients that cannot tolerate oral nutrition, support can be provided *via* naso-enteral feeding or total parenteral nutrition (TPN). To date, no studies have compared these two approaches in the seating of pancreatic pseudocyst and choice is based on availability and local preferences. If one can extrapolate from studies comparing the two modalities in the seating of acute necrotizing pancreatitis, one can expect that jejunal feeding will be related with fewer complications (infection), but may not be able to provide as much calories as TPN.

The rationale of using octreotide as a therapy for pancreatic pseudocyst is that it will decrease pancreatic secretions and aid in pseudocyst resolution. Unfortunately, this strategy has not been rigorously tested and only a handful of case series have been published[30,31].

Most pseudocysts resolve with supportive medical care. Vitas *et al*[32] followed over a period of 5 years 114 patients with the diagnosis of pancreatic pseudocyst. Forty-six patients underwent primary operative therapy, with 13% undergoing emergency operations for pseudocyst-related complications. Although no operative deaths occurred, significant morbidity occurred in 26% of patients (emergency operations, 67%; elective procedures, 10%). The remaining 68 patients were initially treated with a nonoperative, expectant approach. Severe, life-threatening complications in this group (follow-up for a mean of 46 mo) occurred in only six patients (9%); 19 patients eventually underwent elective operation directed at either the pseudocyst or other complications related to pancreatitis. Overall, in patients managed by a nonoperative approach, resolution of the pseudocyst occurred in 57% of the 24 patients with satisfactory radiographic follow-up, with 38% resolving more than 6 mo after diagnosis. Although patients eventually undergoing operation tended to have larger pancreatic pseudocysts than the patients managed successfully nonoperatively (6.9 cm *vs* 4.9 cm), no serious complications occurred in seven patients with pancreatic pseudocysts greater than 10 cm who were treated expectantly[32].

Several studies have indicated that the size of the cyst and the length of time the cyst has been present are poor predictors of potential for pseudocyst resolution or complications, but in general, larger cysts are more likely to become symptomatic or cause complications[33]. However, some patients with larger collections do well; therefore, size of the pseudocyst alone is not an indication for drainage[34,35]. The two main indications for invasive intervention are the presence of symptoms or the presence of complications (infection, bleeding, gastric outlet or biliary obstruction).

DRAINAGE PROCEDURES

Symptomatic pseudocysts or the presence of some complications (infected pseudocyst, gastric outlet or biliary obstruction) are the main two indications for some type of drainage procedure. To date, no prospective controlled studies have compared directly percutaneous, surgical and endoscopic drainage approaches. As a result, the management varies based on local expertise but in general endoscopic drainage is becoming the preferred approach.

Percutaneous drainage

External drainage can be achieved using CT or US guidance. With this technique, a drainage pigtail catheter is placed percutaneously into the fluid cavity and fluid is drained. Three-dimensional ultrasonography has been reported useful for the guidance of catheters into cyst cavities and avoiding vessels[36]. The fluid is collected over several weeks into an external collection system. When the drainage output becomes minimal, the catheter is removed. Contrast injection into the cyst cavity will demonstrate the size of the remaining cavity and this finding can be used to monitor the progress. This technique is successful at resolving pseudocysts, but it has a high risk of infections. The external drain tends to create significant patient discomfort. Furthermore, the catheter tends to clog and may require repositioning and exchange. The reported long-term success rate for pseudocyst resolution for US-guided pseudocyst drainage is around 50%. Unsuccessful drainages are usually caused by large ductal leaks or obstruction of the main pancreatic duct. Percutaneous catheter drainage is contraindicated in patients who are poorly compliant and cannot manage a catheter at home. It is also contraindicated in patients with strictures of the main pancreatic duct and in patients with cysts containing bloody or solid material[37,38].

Surgical drainage

Surgical drainage of pseudocysts is accomplished by providing a communication between the pseudocyst

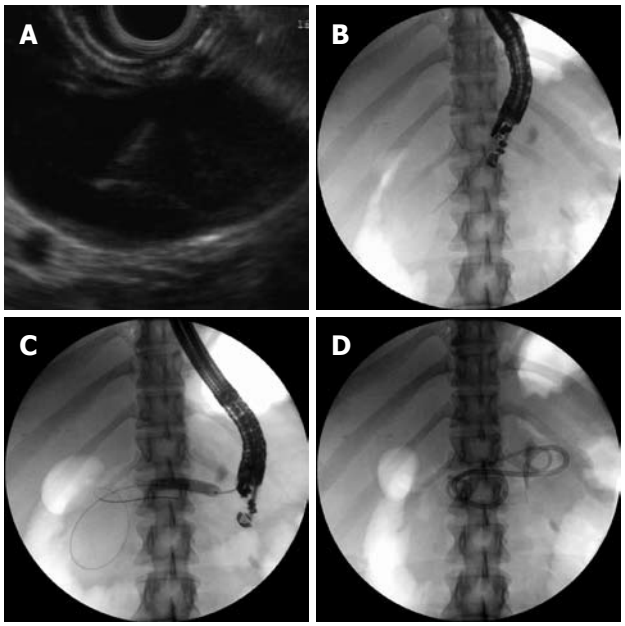

Figure 1 EUS and fluoroscopic image. A: EUS image of pseudocyst with FNA needle; B: Fluoroscopy image of pseudocyst with FNA needle; C: Fluoroscopy image of balloon dilating the cyst gastrostomy tract; D: Fluoroscopic image of two double pigtail stents draining the pseudocyst cavity.

cavity and the stomach or small bowel. This approach to drainage is often reserved for those patients that cannot tolerate or have failed percutaneous or endoscopic drainage. The surgical stoma should be placed in the most dependent portion of the cystic cavity in order to maximize the chances of complete drainage. The stoma usually remains patent and functional for several months.

Adams and Anderson published findings from a retrospective analysis of 94 patients[39]. The study population consisted of 42 patients undergoing internal surgical drainage and 52 patients undergoing percutaneous pseudocyst drainage. Significant complications occurred in 16.7% of the patients undergoing surgery and in 7.7% of the patients undergoing percutaneous drainage ($P > 0.05$). A subsequent operation was required in 9.5% of the surgical group and 19.2% of the percutaneous drainage group ($P > 0.05$). A significantly higher mortality rate was associated with surgical therapy (9%) than with percutaneous therapy (1%) ($P < 0.05$)[39].

Endoscopic drainage

Endoscopic drainage of pseudocysts is becoming the preferred therapeutic approach because it is less invasive than surgery, avoids the need for external drain and has a high long-term success rate. Drainage is accomplished with either a transpapillary approach with ERCP or direct drainage across the stomach or duodenal wall. A transpapillary approach is used when the pseudocyst communicates with the main pancreatic duct, usually in the genue of the pancreatic duct. This approach is also successful for patients with pancreatic duct disruption.

A transgastric or transduodenal approach is used when the pseudocyst is directly adjacent to the gastro-duodenal wall. To determine the size and location of the pseudocyst, and to measure the thickness of the pseudocyst wall, EUS has become the test of choice. A distance between the gastric or duodenal wall and cyst wall of more than 1 cm or the presence of large intervening vessels or varices are relative contraindications for endoscopic drainage[40,41]. Transgastric or transduodenal stenting of pseudocysts may be performed using an endoscopic approach under fluoroscopic guidance or using EUS to introduce the guidewire into the pseudocyst cavity.

The endoscopic approach is dependent upon the presence of a bulge into the lumen of the stomach or duodenum in order to determine the entry site for catheterization. This approach has several inherent risks, including missing the pseudocyst, injuring intervening vessels, and sub-optimal placement of the drainage catheter[42]. Therapeutic echoendoscopes now make it possible to treat pseudocysts with EUS-guided transmural stenting[43]. Several series have described the deployment of a 7 Fr stent that is introduced with a needle knife catheter[44]. A new large-channel echoendoscope allows the use of 10 Fr stents across the stomach or duodenum[45].

The exact technique for transmural pseudocyst drainage has not been standardized. In our institution, we prefer a combined EUS/fluoroscopy guided technique. The linear therapeutic channel EUS endoscope is used to detect an optimal site of apposition of pseudocyst and gut wall, free of intervening vascular structures (Figure 1A). The 19 Fr gauge EUS FNA needle is then advanced into the cyst cavity under real-time ultrasound guidance. The needle position is then located under fluoroscopy (Figure 1B). After the pseudocyst cavity has been entered, fluid is aspirated and a floppy-tip 0.035 guide wire is advanced *via* the needle and under fluoroscopic control is curled few times into the cyst cavity. The cyst-gastrostomy (duodenostomy) fistula tract is then pneumatically dilated, with 8 to 15 mm biliary balloon dilators (Figure 1C). The size of the balloon used for dilation is arbitrarily determined based on the size of the cyst, proximity of vessels, presence of necrotic debris in the cyst cavity, viscosity of the aspirated pseudocyst fluid and the presence of infection. In an attempt to decrease the risk of bleeding we try to avoid using electrocautery to create the fistulous tract. In a rare occasion, when the pseudocyst wall is very tick and the balloon dilator cannot be advanced, we use the Cystotome (Cook Medical, Winston-Salem, NC, USA). We will then stent the tract with two or more double pigtail stents (7F-10F) *via* the EUS scope (Figure 1D).

In a small series, the EUS approach has resulted in a success rate of more than 90% in patients with chronic pseudocysts[46]. The recurrence rate after endoscopic drainage is low, 4%, and the complication rate is less than 16%[47].

EUS is also capable of guiding the drainage of infected pseudocysts using naso-cystic drains[48]. It may even be possible to drain infected necrotic pancreatic tissue using EUS and endoscopic techniques[49].

Hookey *et al*[50] published a chart review and prospective follow-up for 116 patients with attempted

endoscopic drainage of symptomatic pancreatic-fluid collections (pseudocysts and organized pancreatic necrosis). A total of 116 patients presented with fluid collections classified as acute fluid collection (n = 5), necrosis (n = 8), acute pseudocyst (n = 30), chronic pseudocyst (n = 64), and pancreatic abscess (n = 9). The median diameter of the collection drained was 60 mm (15-275 mm). Median follow-up after drainage was 21 mo. The drainage technique was transpapillary in 15 patients, transmural in 60, and both in 41. Successful resolution of symptoms and collection occurred in 87.9% of cases. No difference in success rates was observed between patients with acute pancreatitis and those with chronic pancreatitis. However, drainage of organized necrosis was associated with a significantly higher failure rate than other collections. No significant differences were observed regarding success when disease, drainage technique, or site of drainage was considered. Complications occurred in 13 patients (11%), and there were six deaths in the 30 d after drainage, including one that was procedure related. He concluded that endoscopic drainage of pancreatic-fluid collections is successful in the majority of patients and is accompanied by an acceptable complication rate[50].

Muscatiello *et al*[51] published a case report of alcohol use for the treatment of a pancreatic pseudocyst. In his report, aspiration of the pancreatic pseudocyst was started, and after an apparent reduction in the volume of the pseudocyst by about 30%, 30 mL of absolute ethanol diluted 1:1 with saline was injected and maintained for about 10 min. Aspiration then continued until EUS imaging showed that the cyst was completely empty. CT 24 h later demonstrated no complications and confirmed that the procedure had been successful. Culture of the aspiration fluid identified a *Pseudomonas aeruginosa* and *Citrobacter freundii* complex. Cytological examination did not show any neoplastic cells. The patient was discharged on the seventh day with no symptoms and with normal laboratory tests. It seems that, in addition to causing sclerosis of the cystic wall, ethanol contributes to sterilizing the infected fluid collection. In that case, a long follow-up period (18 mo) in which there was no recurrence of the pseudocyst confirms that this procedure may be useful in the treatment of organized necrotic abscesses and pancreatic abscesses when there is no communication with the pancreatic duct[51].

In a large retrospective analysis of 603 patients who were undergoing EUS-FNA of pancreatic cysts, possible infection developed in only a single patient. The majority of patients in this series (90%) received antibiotic prophylaxis, most commonly a fluoroquinolone given for 3 d after the procedure, and this may possibly explain the low infection rate. The benefit of prophylactic antibiotics before an FNA of cystic lesions has not been evaluated by prospective randomized studies[52].

The ASGE, in 2008, published the guidelines for prophylactic use of antibiotics for GI endoscopy. According to these guidelines, prophylaxis with an antibiotic, such as a fluoroquinolone administered before EUS-FNA of cystic lesions along the GI tract including pancreatic cyst. Antibiotics may be continued for 3 to 5 d after the procedure (supported by observational studies). When antibiotic prophylaxis is administered, a fluoroquinolone administered before the procedure and continued for 3 d after the procedure is a reasonable regimen[53].

Cahen *et al*[54] published a retrospective study to evaluates the short-term and long-term results with the endoscopic drainage of pancreatic pseudocyst and aimed to identify procedural modifications that may improve its safety and efficacy. A total of 92 patients were included (66 men, 26 women; median age 49 years). The technical success rate of the drainage procedure was 97% and the mortality rate was 1%. Complications occurred in 31 patients (34%), eight of which (9%) were major and required surgery: hemorrhage in four cases (three of which were caused by erosion of a straight endoprosthesis through the cyst wall), secondary infection in three, and perforation in one. During a median follow-up period of 43 mo, 10 patients (11%) underwent additional (nonendoscopic) treatment for a persistent cyst and five (5%) for a recurrent cyst. Overall, endoscopic drainage was successful in 65 patients (71%). He concluded that endoscopic drainage is an effective treatment for pancreatic pseudocysts and offers a definitive solution in almost three-quarters of the cases. The majority of major complications might have been prevented by using pigtail stents instead of straight stents and by taking a more aggressive approach to the prevention and treatment of secondary cyst infection[54].

COMPLICATIONS OF PANCREATIC PSEUDOCYST

Splenic complications

Splenic complications of pseudocyst include massive hemorrhage into the pseudocyst, sepsis with splenic infarction, and splenic vein thrombosis. The diagnosis of intrasplenic pseudocyst, based on clinical findings alone, is difficult to arrive at but should be suggested by the presence of a mass in the left upper quadrant. Sonography and computerized axial tomography may be particularly helpful in confirming splenic involvement. Selective celiac arteriography should be performed whenever splenic involvement is suggested in order to confirm the diagnosis and to search for pseudoaneurysm formation. Urgent surgical intervention is usually warranted in view of the high incidence of serious complications and the propensity toward rapid clinical deterioration. Resection of the pseudocyst by splenectomy and distal pancreatectomy is the treatment of choice[55].

Rupture

Rupture of a pseudocyst can have either a favorable or an unfavorable outcome and this depends on whether it ruptures into the gastrointestinal tract, into the general peritoneal cavity or into the vascular system[56,57]. Rupture into the gastrointestinal tract either results

in no symptoms or leads to melaena or hematemesis that usually requires urgent measures. Rupture into the general peritoneal cavity results in features of peritonitis and occasionally hemorrhagic shock. Emergent surgical exploration is usually required. While an internal drainage should always be aimed for, usually a thorough abdominal lavage and external drainage is all that can be achieved safely.

Hemorrhage

Hemorrhage can greatly complicate the course of a pseudocyst[58]. The morbidity and mortality is very high because it can appear without warning and is usually due to erosion of a major vessel in the vicinity of the pseudocyst. Interventional radiology can play an invaluable role both in locating the source of bleeding and in embolisation of the bleeding vessel[59]. Without prior information of the bleeding point, surgical exploration can be hazardous and challenging.

Infection

Infection occurs either spontaneously or after therapeutic or diagnostic manipulations. While infected pseudocyst can initially be treated with conservative means, a majority of patients will require intervention. Traditionally surgery has been the preferred modality but endoscopic treatment is gaining acceptance[48,60]. An external drainage may be necessary in selected situations such as when there is evidence of gross sepsis and the patient is too unstable to undergo surgical or endoscopic drainage.

Biliary complications

Biliary complications occur due to a large cyst in the pancreatic head region obstructing the common bile duct and resulting in obstructive jaundice[61,62]. Therapeutic endoscopy with short-term biliary stenting is valuable in this situation. It can be retained until either the pseudocyst resolves or is treated by intervention.

Portal hypertension

Portal hypertension can result from compression or obstruction of the splenic vein/portal vein either by the cyst alone or in conjunction with underlying chronic pancreatitis[63]. In this situation, surgery appears to be the only treatment modality available and an appropriate surgical procedure can effectively treat this form of portal hypertension.

CONCLUSION

Pancreatic pseudocysts are the result of acute or chronic pancreatitis and are the most common cystic lesions of the pancreas, accounting for 75%-80% of such lesions. The most common symptoms are abdominal pain, nausea and vomiting, although they can be asymptomatic. Abdominal CT is an excellent choice for initial imaging. EUS plays an important role in differentiating pseudocyst from other cystic lesions of the pancreas and can greatly assist in transmural endoscopic drainage. Initial management consists of supportive care. Persistent symptoms and the development of complications warrant invasive intervention. The surgical, percutaneous and endoscopic pseudocyst drainage procedures have not been directly compared in high quality prospective randomized studies and the preferred approach varies based on patient preferences and local expertise. In recent years, the endoscopic approach has gained popularity with surgery reserved for patients who had failed endoscopic or percutaneous drainage. A tailored therapeutic approach taking into consideration patient preferences and involving multidisciplinary team of therapeutic endoscopist, interventional radiologist and pancreatic surgeon should be considered in all cases.

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GUIDELINES CLINICAL PRACTICE

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Cystic neoplasms of the pancreas: A diagnostic challenge

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Abstract

Cystic neoplasms of the pancreas are increasingly recognized due to the expanding use and improved sensitivity of cross-sectional abdominal imaging. Major advances in the last decade have led to an improved understanding of the various types of cystic lesions and their biologic behavior. Despite significant improvements in imaging technology and the advent of endoscopic-ultrasound (EUS)-guided fine-needle aspiration, the diagnosis and management of pancreatic cystic lesions remains a significant clinical challenge. The first diagnostic step is to differentiate between pancreatic pseudocyst and cystic neoplasm. If a pseudocyst has been effectively excluded, the cornerstone issue is then to determine the malignant potential of the pancreatic cystic neoplasm. In the majority of cases, the correct diagnosis and successful management is based not on a single test but on incorporating data from various sources including patient history, radiologic studies, endoscopic evaluation, and cyst fluid analysis. This review will focus on describing the various types of cystic neoplasms of the pancreas, their malignant potential, and will provide the clinician with a comprehensive diagnostic approach.

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Key words: Cystic neoplasm; Endoscopic ultrasound; Pancreas; Pancreatic cyst; Pancreatitis

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INTRODUCTION

Pancreatic cystic neoplasms, despite increased recognition, remain rare and represent approximately 10%-15% of primary cystic masses of the pancreas[1-3]. Many pancreatic cystic masses are discovered incidentally during the work-up for abdominal pain, diarrhea, and other non-specific gastrointestinal symptoms and represent a frequent clinical referral in tertiary academic centers with pancreatic expertise. Not surprisingly, the increase in the diagnosis of a pancreatic cystic mass parallels that of the improved number and type as well as the improved overall sensitivity of cross-sectional imaging studies used in routine practice today[4]. It is important for today's practicing physician to be aware of these increasingly recognized neoplasms on radiological imaging, and more importantly, to understand the potential for the presence or development of pancreatic malignancy in a certain subset of these lesions.

CLASSIFICATION

The classification of cystic pancreatic neoplasms has its roots in the surgical, radiological, and perhaps most importantly in the clinical pathological literature, and dates from the mid to late 1970s[5,6]. The distinction between serous and mucinous cystic neoplasms (MCNs) was first realized at that time and despite many modifications and attempts at radiological[7], endoscopic[8], and more recently with newer laboratory-based analysis using techniques such as mass spectrometry[9], remains intact and a solid initial clinical approach to these neoplastic lesions even today. Importantly, our understanding of MCNs has evolved and since the early 1980s, the clinical entity we now recognize as intraductal papillary mucinous neoplasm (IPMN) was first described in the literature[10]. IPMN remains a very important "lesion of clinical distinction" when evaluating pancreatic cystic neoplasms and is recognized as a distinct histopathological entity as evidenced by the World Health Organization histological classification system[11] (Table 1).

Table 1 Histological classification of pancreatic cysts

	Histologic classification
Serous cystic tumors	SCA
	Serous cystadenocarcinoma (rare)
Mucinous cystic tumors	Mucinous cystadenoma
	Mucinous cystadenoma with moderate dysplasia
	Mucinous cystadenocarcinoma
	Noninfiltrating
	Infiltrating
Intraductal papillary mucinous tumors	Intraductal papillary mucinous adenoma
	IPMN with moderate dysplasia
	Intraductal papillary mucinous carcinoma
	Noninfiltrating
	Infiltrating
Solid pseudopapillary tumors	

MALIGNANT POTENTIAL OF PANCREATIC CYSTIC NEOPLASMS

The incidentally discovered pancreatic cystic neoplasm not only represents an alarming clinical discovery, but for the affected patient, in many instances, represents a pre-cancerous condition with a great deal of uncertainty regarding management. The discussion regarding malignant potential focuses mainly on the distinction between IPMN and MCNs. Serous cystadenomas (SCAs) are largely benign lesions although case reports of malignant transformation do exist and as such are often managed non-surgically. Solid pseudopapillary tumors have a fairly well defined behavior and malignant risk and are often managed surgically.

The distinction between IPMN lesions and MCN lesions remains a controversial topic and relies on several clinical and pathological factors. Clinical factors include patient age, location of the cyst, cyst characteristics, and relationship to the main pancreatic duct. As is described in more detail below, IPMNs are found most often in male patients in their 60s or 70s, and are more often than not found in the pancreatic head/neck region. IPMNs appear "grape-like" on imaging, including on endoscopic ultrasound (EUS) and appear as cysts side by side one another rather than the "Cyst within a cyst" characteristically seen in MCNs. IPMN lesions also communicate with the pancreatic duct, a feature not seen in MCNs. MCNs in comparison, are often seen in females in the 40 to 50-year age range and are located most often in the pancreatic body and tail regions.

Pathologically, the best studied differentiation criteria involve the presence of ovarian-type stroma on histological analysis[5,12]. The presence of ovarian-type stroma is strongly suggestive of an MCN lesion, although non-ovarian stroma MCNs have been reported in the literature. The distinctions between MCN and IPMN lesions are clinically important as the malignant potential and resultant management are often times based on these differences and permit an individualized care plan, rather than pursuing a "remove all mucinous neoplastic process" management style.

The malignant potential of the various cystic neoplasms of the pancreas are important for the given clinician and are best understood by dividing IPMNs into main-branch *vs* side-branch lesions and comparing/contrasting these with the MCN. Main branch IPMN lesions carry the highest percentage of malignancy, ranging in most studies between 60% and 92%[13-16]. Invasive malignancy defined as non-carcinoma-in-situ is also more common in these lesions and approaches 60% in some studies. Side-branch IPMN lesions in comparison are less often malignant, with a range of malignancy in reported studies between 6% and 46% and are less likely to be invasive, with the highest reported percentage in the 30% range[17,18]. In comparison to IPMN lesions, MCNs have a malignant potential ranging from as low as 6% to as high as 36%[19-21]. A better understanding of the malignant potential of MCN lesions is likely to improve with further acceptance of the ovarian-type stroma as diagnostic criteria regarding these lesions.

PRESENTATION/EPIDEMIOLOGY

The exact prevalence of pancreatic cysts is difficult to measure because many patients will be entirely asymptomatic, but it has been estimated to be approximately 20% in patients undergoing radiological imaging for non-pancreatic diseases/indications[22]. The asymptomatic nature of these cystic lesions (estimated at 40%-75%) in some studies[23] make further epidemiological studies a clinically difficult task. An autopsy series from Japan estimated the prevalence of pancreatic cysts to be 25%, with an increasing prevalence paralleling advanced patient age[24]. Regardless, the proportion of pancreatic cysts felt to be primary cystic neoplasms is well documented and in the range of 10%-15% with the remaining majority of cysts found to be pseudocysts[25]. This percentage draws attention to the importance of ruling out the presence of a pancreatic pseudocyst using a combination of historical questioning, and in many cases of cystic sampling, usually done *via* EUS.

DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS OF PANCREATIC CYSTIC LESIONS

Once the presence of a pancreatic cyst has been established by an imaging modality, the cornerstone of management is to differentiate between a pseudocyst and a cystic neoplasm. If a pseudocyst has been effectively excluded, a prudent clinical strategy regarding pancreatic cysts is the division into serous *vs* mucinous neoplasms. During the evaluation of a pancreatic cyst, it is important for the clinician to have an understanding of the different cyst types, their typical location in the pancreas, and their biological behavior. Serous cystic neoplasms (SCNs) represent approximately 30% of primary cystic neoplasms of the pancreas[26], with the largest subset being SCAs. The mucinous neoplasms

Table 2 Typical characteristics of pancreatic cystic lesions

Cyst type	Pseudocyst	SCA	MCN	IPMN	SPN
Age	Variable	Middle-aged	Middle-aged	Elderly	Young
Sex	M > F	F > M	Female	M > F	Female
Pancreatitis history[1]	Yes	No	No	Yes[2]	No
Location	Evenly	Evenly	Body/tail	Head	Evenly
Malignant potential	None	Rarely	Moderate to high	Low to high	Low
Biliary obstruction	Yes, Uncommon	No	No	Yes, Uncommon	No

SCA: Serous cystadenoma; MCN: Mucinous cystic neoplasm; IPMN: Intraductal papillary mucinous neoplasm; SPN: Solid pseudopapillary neoplasm. [1]A history of pancreatitis episodes and pancreatic risk factors including alcohol abuse, gallstones and complications, or family history of pancreatitis is often given; [2]Pancreatitis due to IPMN is predominately of the main pancreatic duct subtype.

are primarily subdivided into MCNs which represent approximately 45%-50% of primary cystic neoplasms of the pancreas[26], and IPMNs which make up approximately 25% of primary cystic neoplasms[27].

It is of great clinical importance at this point of the work-up to consider the clinical background of the patient with a newly discovered pancreatic cystic neoplasm. Remembering that a large proportion of pancreatic cysts are found to be pseudocystic in nature, a thorough review of the history for episodes of definable pancreatitis in conjunction with risk factors for pancreatitis, such as chronic alcohol ingestion, family history of "pancreatic diseases" as often described by patients and their families, and autoimmune disease is always a good clinical starting point. A clear history of a well documented episode of pancreatitis strongly suggests that the cystic pancreatic lesion is a pseudocyst, but occasionally an attack of pancreatitis will be the clinical presentation of a neoplastic cystic lesion particularly an IPMN[28]. Patient demographics including age, sex, and presence or absence of symptoms and the location of the cyst are important considerations while a diagnosis is being sought. For example, MCNs tend to be a middle-age, female-predominant disease with most, but not all, lesions located in the pancreatic body or tail[20]. Serous cystic adenomas (SCAs) in contrast, while present most often in middle-aged females are evenly distributed throughout the pancreatic gland, while IPMNs have an elderly male predominance and are usually located, but not confined to the pancreatic head region[29,30]. Solid pseudopapillary tumors of the pancreas (SPNs) remain a pathologically distinct, rare clinical entity occurring predominately in young females[31]. A comparative index involving the different pancreatic cystic neoplasms as well as the pancreatic pseudocysts is shown in Table 2, and the usual location of pancreatic cystic lesions in Figure 1[32].

The discovery of a lesion thought to represent a possible pancreatic cystic neoplasm is often made incidentally by computed tomograghy (CT) scanning performed for other clinical reasons. With this in mind,

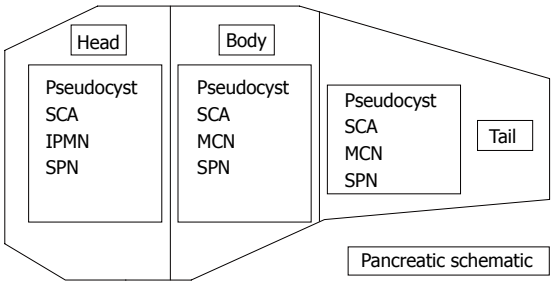

Figure 1 Typical location for pancreatic cystic lesions.

a thorough understanding of the different imaging modalities, both radiological and if available endoscopic, is needed to best construct a diagnostic algorithm for optimum care for these patients. The availability of endoscopic retrograde cholangiopancreatography (ERCP) and perhaps most importantly, EUS plus/minus fine-needle aspirate (FNA) and cystic fluid analysis, has led to a much improved understanding and characterization of these lesions.

RADIOLOGICAL IMAGING STUDIES

Traditionally, three imaging modalities have been used to evaluate pancreatic lesions: trans-abdominal ultrasound (US), CT scanning, and magnetic resonance imaging (MRI)/magnetic resonance cholangiopancreatography (MRCP). Trans-abdominal US, while having the advantage of being inexpensive and readily available, is very operator-dependent, and is limited in its ability to visualize the entire pancreas. Furthermore, the presence of significant bowel gas limits the sensitivity of US for characterization of pancreatic cystic processes.

CT scanning, particularly with intravenous contrast enhancement, is a widely available, relatively inexpensive imaging modality and is often the first imaging procedure ordered when a diagnosis of a pancreatic cystic neoplasm is considered. A review of the diagnostic accuracy of CT scanning has recently been performed[32] with a reported range between 20% and 90%. Differences in study design, characterization of lesions, especially those with atypical features[33,34], and the ultimate study goal, i.e. specific cyst type[34-37] *vs* differentiation of benign *vs* malignant cyst types[8] all were felt to contribute to the wide range in diagnostic accuracy.

The typical appearance of a given cystic neoplasm is reported in many ways *via* CT. Size (i.e. microcystic (< 2 cm) *vs* macrocystic (> 2 cm), uni- *vs* multilocularity, pancreatic duct communication and/or dilation, and the presence of a mass or mural nodule remain the most important imaging characteristics seen on routine CT. SCAs are characteristically microcystic with many small cysts within the larger cyst creating a "honeycomb" type pattern. A central stellate scar is often seen at the center of an SCA and is considered pathognomonic. Pancreatic duct communication is rarely seen and dilation of the pancreatic duct also remains uncommon. MCNs are in comparison, most often macrocystic, although

microcystic lesions do occur and characteristically are multilocular with an "orange fruit" type appearance. Dilation of the pancreatic duct is uncommon as is communication with the main pancreatic duct. IPMN lesions in contrast, are often described as a "bag of grapes" and contain numerous smaller cysts. Pancreatic duct communication is common, and in main branch IPMN lesions, pancreatic duct dilation is seen and predictive of an invasive nature. Associated mural nodules and/or masses are most often observed in IPMN lesions and to a lesser extent in MCNs. The presence of a mural nodule is significant, as this is often predictive of an invasive cystic neoplasm.

MRI of the abdomen when combined with MRCP is a rapidly emerging imaging modality with widespread availability, and has great potential to add to our understanding of pancreatic cystic lesions. MRI/MRCP comparatively, in relation to other imaging modalities, is rivaled only by EUS in its ability to obtain quality images of not only the pancreatic parenchyma, but also of the pancreatic and biliary ductal structures[38-41]. MRCP does remain inferior to ERCP in terms of diagnostic accuracy, but the gap is narrowing and MRCP offers a non-invasive means of diagnosis compared with ERCP and its complications, most notably post-ERCP pancreatitis.

ENDOSCOPIC STUDIES

The role of endoscopy, specifically ERCP and EUS, in the evaluation and diagnosis of pancreatic cystic neoplasms is a study in evolution that continues today. ERCP remains the most sensitive diagnostic modality for detecting communication between the main pancreatic duct and a given cystic lesion[42,43]. Additionally, in a minority of cases an endoscopic diagnosis of an IPMN can be established if a patulous papilla with mucin extrusion, also sometimes referred to as the "fish-eye" ampulla is visualized[30]. The use of ERCP as a primary diagnostic tool in pancreatic cystic neoplasms is not routinely recommended. In most cases, the correct diagnosis can be achieved with a higher yield, less invasive test.

EUS, since its introduction as an endoscopic technology in the late 1970s and early 1980s, has become an increasingly available tool in the diagnosis, management, and in some cases, therapy of pancreatic cystic neoplasms. The ability to better describe/characterize pancreatic cystic neoplasms, in particular those lesions thought to be pre-malignant or frankly malignant, make the use of EUS, both with and without FNA, an attractive option in the cystic neoplastic work up. EUS criteria for mucinous/malignant neoplasms is still evolving, but include size greater than 2 cm, pancreatic duct dilation, the presence of wall calcifications, and perhaps most importantly the presence of a frank mass or mural nodule. Despite initial enthusiasm, however[44], numerous studies[45-49] have demonstrated a wide range of diagnostic accuracy for EUS imaging alone ranging from 40%-96%. While many factors including study design, number of patients enrolled, goals of a particular study, and interobserver EUS agreement contribute to this discrepancy, it is important to note that a single, prospective study[49] achieved a diagnostic accuracy of approximately 51%. Clearly, larger, prospective, multi-center studies are needed to better define the role of EUS in the diagnostic work up of a pancreatic cystic neoplasm.

EUS, in addition to its imaging capabilities outlined above, allows direct sampling of cystic contents and the cyst wall in an effort to better determine the type of cyst present. The performance of FNA does, however, remain limited to larger, tertiary centers with extensive experience in EUS. In addition, analysis of cystic fluid is often subject to local cytological and laboratory expertise, with a definite learning curve present for accurate analysis of cystic contents and in some cases, by a small volume of aspirate obtained at FNA.

Ideally, an aspirated pancreatic cystic neoplasm should be evaluated for both cytological diagnosis and for the presence of specific intracystic proteins such as amylase and carcinoembryonic antigen (CEA). The cytological evaluation includes specific testing for the presence of columnar epithelial cells which stain for mucin (MCNs, IPMNs), or cuboidal epithelial cells which stain for glycogen (SCAs). Several studies have appeared in the literature regarding the analysis of pancreatic cystic fluid. Several larger studies involving cystic fluid cytological analysis[32,50,51] reflect a sensitivity of approximately 50%, a low but reproducible percentage, while a more recent study by Moparty *et al*[52] revealed a cytological sensitivity of approximately 93% in the differentiation of mucinous and non-mucinous pancreatic neoplasms. The cytological analysis of cystic fluid continues to be an area of intense research.

Amylase level is routinely checked in the cyst fluid aspirate and may be of some diagnostic value. It is uniformly elevated in pseudocysts and IPMNs and frequently elevated in MCNs, but consistently low in SCAs. The analysis of specific intracystic, aspirated proteins continues to be an evolving process. Several proteins including CA19-9, CEA, CA-125, and CA72-4 have been studied. The best studied and currently used most often in routine practice is the level of CEA. The basic differentiation involving CEA level is between the lesions which are mucinous (usually, but not always elevated CEA levels) and those which are serous (low CEA levels)[51-54]. A low CEA level (i.e. < 5 ng/mL) has been shown in pooled data[44,48,51,55], to have a sensitivity between 50%-100% and a specificity of 77%-95% to differentiate between mucinous and serous lesions. The CEA level required to best distinguish a mucinous from a serous lesion continues to be debated in the pancreatic literature, with CEA cutoff levels deemed diagnostically sensitive in the range 20 to 800[48,49,55-57]. The wide range of reported CEA levels lends confusion to the analysis of cystic pancreatic fluid. It must be remembered, however, that by increasing the cutoff value of the CEA level considered diagnostic for mucinous lesions, the specificity of the test increases at the expense of decreased sensitivity. Currently, no standardized cutoff

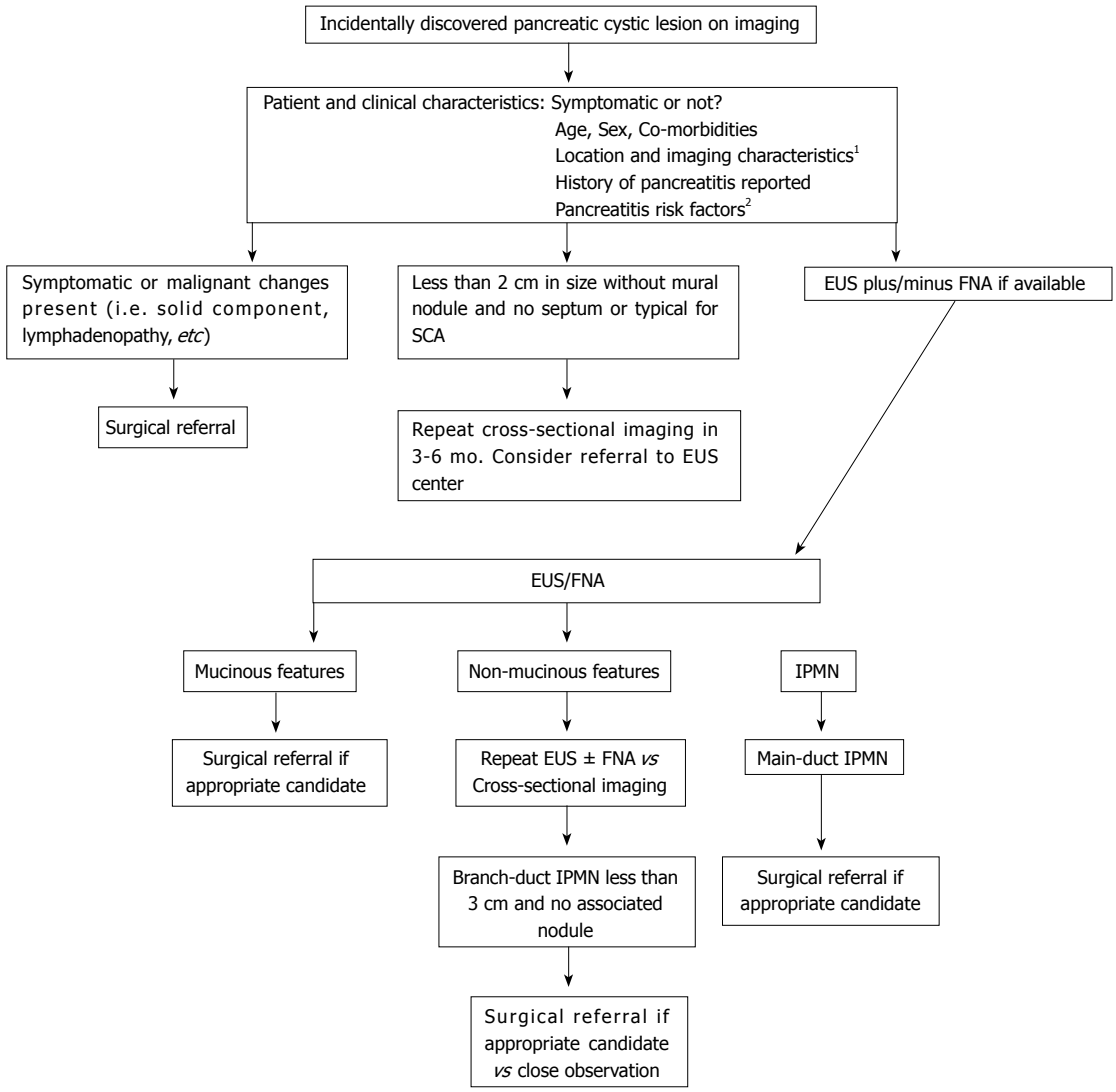

Figure 2 Diagnostic algorithm. [1]Imaging characteristics include size > 2 cm, mural nodule presence/absence, total cyst number, pancreatic duct communication. [2]Pancreatitis risk factors include alcohol use, family history of pancreatitis, autoimmune diseases.

level for CEA exists, however, many centers, particularly in the US, use a CEA level of 192 ng/mL, as established by Brugge *et al*[49] as diagnostically sensitive (75%) and specific (84%). At present, aspirated cystic fluid should be evaluated for cytological and biochemical analysis. The biochemical tests which should be routinely ordered are CEA level and amylase. If insufficient fluid is available (e.g. small cyst or very viscous fluid), CEA level should be obtained first with cytology and amylase level ordered only if there is a sufficient amount of fluid left for analysis.

DIAGNOSTIC ALGORITHM

As outlined above, the diagnosis and prospective management of a pancreatic cystic neoplasm involves coordination on several levels, ranging from the initial discovery to possible surgical referral if a frankly malignant or pre-malignant pancreatic cystic neoplasm is suspected. A proposed diagnostic algorithm beginning with the incidentally discovered pancreatic lesion is presented in Figure 2.

CONCLUSION

The evaluation of cystic lesions of the pancreas remains a process in evolution. Significant advances have been made in expanding our understanding of these lesions and in the refinement of our diagnostic approach. A comprehensive diagnostic strategy which incorporates data from patient history, lesion imaging, EUS, and cyst fluid analysis will provide an accurate diagnosis in most cases.

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ERCP wire systems: The long and the short of it

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Abstract

Guidewires are routinely used at the time of endoscopic retrograde cholangiopancreatography (ERCP) to gain and maintain access to the desired duct and aid in the advancement of various devices. Limitations of the traditional long-wire systems have led to the introduction of three proprietary short-wire systems. These systems differ in many respects but share two main principles: They lock a shorter wire in position to allow advancement or removal of various devices without displacement of the wire and they all allow for physician control of the wire. In this comprehensive review, we describe the key features of the three currently available short-wire systems: RX, Fusion and V systems. We also focus on the potential benefits and drawbacks that accompany the short-wire concept as a whole and each specific system in particular. Although the available data are limited, it appears that the use of the short-wire systems lead to reduced procedure, fluoroscopy and device exchange times, decreased sedation requirements, improved wire stability and increased endoscopist control of the wire. Furthermore, the physician-controlled wire-guided cannulation has the potential to decrease ampullary trauma and the rate of post-ERCP pancreatitis. The short guidewire systems appear to be an improvement over the traditional long-wire systems but further studies directly comparing the two approaches are needed.

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Key words: Endoscopic retrograde cholangiopancreatography; Guidewires; V-system; RX system; Fusion system

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INTRODUCTION

Guidewires are an essential part of both diagnostic and therapeutic endoscopy. They are used to gain or maintain access into a lumen or cavity. In addition, they have an integral role in the advancement of a variety of devices[1]. Guidewires vary in material, length, diameter, and design to aid in specific situations encountered by the endoscopist during a procedure.

The construction of guidewires is designed for use depending on their individual qualities. Monofilament wires are made with stainless steel and are used for their rigidity[1]. Coiled wires have an inner monofilament core which has the quality of stiffness accompanied by an outer spiral coil encompassing durability and flexibility[1]. Lastly, coated or sheathed wires have a monofilament core made of stainless steel, nitinol, or other alloys, whereas the outer sheath may be made of teflon (DE), polyurethane, or another polymer[1]. Many wires have platinum or tungsten dipped cores to enhance visualization during fluoroscopy. Tips have various designs such as straight, angled, J-shaped, or tapered. Wires can range from 150-650 cm in length and from 0.46-0.97 mm in diameter. A common source of confusion pertaining to endoscopic retrograde cholangiopancreatography (ERCP) wires is the fact that it is customary to display the length of the guidewire in cm and the diameter in inches.

Traditional guidewire usage requires advancement under direct visualization through the endoscope with or without fluoroscopy. Maintenance of the guidewire

position is essential to the safety of dilating procedures and tube placements. Assistance with printed markers and movement guides on the wire can decrease the risk of displacement[2].

Applications for guidewire use in the gastrointestinal (GI) tract are vast. These include advancement of dilators, esophageal stents, manometry catheters, feeding tubes, colonic stents, stricture dilatation and endoscopic ultrasound (EUS)-guided biliary and pancreatic access[1]. Guidewires are an indispensable part of ERCP and are used to gain and maintain access to the desired duct, and provide a platform for insertion or withdrawal of various devices.

CHALLENGES WITH LONG GUIDEWIRE ERCP SYSTEMS

Traditionally, long wires were exclusively used at the time of ERCP. The long length is dictated by the need to exchange various devices over the wire. The length of a typical long wire used for ERCP ranges from 420 to 480 cm. This excessive length creates a number of problems. Since the assistant is in control of the wire and the physician is in control of the ERCP device, excellent communication between the physician and assistant is required. Failure to do so can lead to loss of access, more difficult cannulation, and problems with advancing the wire to the desired target. Furthermore, it is not uncommon for the end of the long wire to touch the floor leading to contamination. Finally, the assistant is challenged to perform multiple tasks at the same time including advancing or retracting the wire, injecting dye, operating the device (inflating/deflating the balloon, flexing/relaxing the sphincterotome *etc*) and doing all of that while making sure that the end of the wire does not touch the floor. Hydrophilic wires are even more prone to displacement from ducts and strictures, which may further contribute to difficulties with catheter exchanges[1]. Repositioning or loss of access to either the bile or pancreatic duct may lead to increases in procedure duration, radiation exposure to patients and staff, failure to place a stent and even perforation[3].

BACKGROUND OF SHORT GUIDEWIRE ERCP SYSTEMS

At the time of ERCP the use of long guidewires is dictated by the need to exchange various devices over the wire. Therefore, the length of the wire should be, at a minimum, twice the length of the device. Recently, advances in catheter technology combined with wire locking systems provided for the development of short-wire ERCP systems. The main feature of all short-wire ERCP systems is the ability to lock the short-wire in position to allow advancement or removal of various devices without displacement of the wire. In a single-center pilot study, Beilstein *et al*[3] have shown that using a prototype duodenoscope (XTJF-140V2F; Olympus America Corp., Melville, NY., USA) led to a shorter exchange time when compared to standard duodenoscopes, and attributed the benefit to the guidewire locking system and fewer instances of repositioning. This milestone study demonstrated that fixation of the guidewire by the endoscope elevator can substantially improve device exchanges over a shorter wire length. However, additional goals of this new innovation included maintaining ductal access, decreasing procedure time, and reducing sedation and fluoroscopy exposure[3]. Endoscopy assistants are and have been essential to the successful use of ERCP, but the ability of the endoscopist to independently manage the guidewire and the scope was considered an advantage during these procedures.

The emergence of short-wire systems evolved in order to counter limitations of the traditional long wires. All short-wire systems share three independent elements including a means of locking the wire in position during a device exchange, exchanging devices over short-wires while maintaining access and decreasing wire lengths between 185 and 270 cm[4]. Device exchanges over a short guidewire are possible by either fixation of the wire externally at the biopsy port or internally at the elevator. Both external and internal lock designs allow physician control of the guidewire at or near the biopsy port. However, the external and internal lock designs differ in many ways. The external lock design uses suction port caps and allows fixation of the wire with all maneuvers except the limited insertion or withdrawal of the leading short-track portion of the device past the biopsy port[4]. In contrast, the internal lock system can be used with either short or long wire devices. When locking occurs at the level of the ERCP scope elevator, assistant control of the wire is needed when devices are passed beyond the elevator.

TYPES OF SHORT GUIDEWIRE ERCP SYSTEMS

There are three available short-wire ERCP proprietary systems. These systems integrate cannulation, sphincterotomy, balloon extraction, balloon dilation and biliary stenting devices. The same short-wire devices can be used with traditional long wires if needed.

The RX system, (Boston Scientific Corporation, Natick, MA, USA) was the first short-wire system introduced in 1999. This system incorporates three components. The RX Locking Device has an external lock that may accommodate fixation of one or two wires. A special biopsy port cap minimizes any air or bile leakage during the procedure. RX Compatible Biliary Devices include both open "tear away" channel monorail designs used with sphincterotomes and catheters, as well as short-track designs used with cytology catheters, stone extraction balloons, dilating balloons and stents. The last component includes the 260-cm long 0.035-inch or 0.025-inch wide Jagwire with a coated firm shaft, flexible hydrophilic leading tip and two colored markings, which aid in detection of wire movement[4]. Use of the 0.035-inch Jagwire, in conjunction with an ultra slim upper endoscope (GIF-XP 160, Olympus America,

Table 1 Differences between the three short-wire systems

Characteristics	RX system	Fusion system	V-system
Type of endoscope	Standard	Standard	V-scope
Type of lock	External at the biopsy port	External at the biopsy port	Internal lock design
Type of device	Open channel tear-away	Close channel breakthrough	Close lumen device
Short–track technology	Yes	Yes	No
Wire length	260 cm	185 cm	270 cm
Can be used with standard guidewires	Yes	Yes	Yes
Can be used with 0.025″ or 0.018″ or angled wires	No	Yes	Yes
Can be used with hydrophilic Glidewire	No	Yes	Yes
Ability to flush wire channel	No	Yes	Yes
Intraductal exchange ability	No	Yes	No
Insertion of multiple stents without the need to recannulate	No	Yes	No
Physician control of wire	Yes	Yes	Yes
Pushability of short-wire devices[1]	++	+++	+++

[1]author own experience.

Center Valley, PA., USA), for maintaining access allowed for direct visualization of the biliary tree to aid in intraductal diagnosis and treatment[5]. The RX system does allow for long wire conversion in appropriate cases with a 200-cm wire attachment. The 0.025-inch and 0.035-inch diameter wire should be used with their respective devices which are not interchangeable.

The Fusion system (Cook Endoscopy; Winston Salem, NC., USA) was introduced in 2004. As in the RX Biliary system, the Fusion system incorporates both short-track and tear-away capabilities. The external wire lock fits on the biopsy port, which enables the locking of one or two wires. A key feature of this system includes a side port that has been placed at 6 cm from the distal tip of any catheter and a closed tear-away channel running the length of the catheter (as opposed to the open tear-away channel of the RX). The availability of a side port near the device tip allows for a true intraductal exchange. With the intraductal exchange, the wire can be disengaged from the device while both are still within the biliary or pancreatic ducts. The device then can be withdrawn while the wire remains in place. Short-track Fusion push catheters are available for both 5F and 7F stents. The Fusion Guidewire, although not extendable, is 0.035 inches in diameter and 185 cm in length with similar features to the Jagwire[4]. Studies from Europe and the US have shown improved placement of multiple stents into the bile duct, or pseudocyst cavity minimizing the number of guidewires used and shortening procedure duration[6,7]. The ability to move from the short-wire system where the physician has control of the wire to the long wire system where there is reliance on an assistant at any point during the procedure is a real advantage of the Fusion system. This system also provides compatibility with all other systems including all hydrophilic wires such as the Glidewire (Boston Scientific Corporation) available commercially.

The V-system (Olympus, Tokyo, Japan) was introduced in 2005. This is the first modification of a duodenoscope for facilitation of wire exchanges[8]. The V-system scope elevator lever includes a V-shaped groove and an increased angle of articulation in comparison to the standard Olympus TJF-160 series endoscope. This design promotes securing and locking of the short guidewire at the elevator level to reduce repositioning of the guidewire during accessory exchanges[8]. The groove described above acts as the internal wire lock allowing use of a catheter without a short-wire track. The V-system devices are similar to the traditional long wire devices at the leading end but have a different design component at the external end[4]. Device manipulation may be simplified by the LinearGuideV, a 0.035-inch diameter, 270 cm long wire with a hydrophilic coating over the leading 50 cm[4]. Spiral markings have been placed starting at 130 mm from the distal end, extending to the proximal end for easier attachment of the LinearGuideV into the V-Groove. The C-Hook allows the device handle to be attached to the V-Scope. This enables the endoscopist to maneuver the guidewire, inject contrast and manipulate the device handle while keeping a grip on the device control section. The main advantage of the C-Hook is that it is very easy for the endoscopist to relinquish control of the guidewire back to the assistant if needed. The main differences between the three available short-wire ERCP systems are summarized in Table 1.

BENEFITS OF SHORT GUIDEWIRE SYSTEMS

One of the main benefits of the short-wire systems is clearly associated with the ability to permit physician-controlled guidewire cannulation of the desired duct. Cannulation is the essential initial step during ERCP and can be challenging for the endoscopist. Median time to successful cannulation was shown to be shorter in a wire-assisted cannulation compared to cannulation achieved after first injecting contrast (120 s *versus* 150 s) ($P = 0.73$)[9]. When used by an experienced endoscopist, Katsinelos *et al*[10] showed that use of a 0.035-inch Jagwire provided an 81.4% success rate for deep common bile duct cannulation *versus* 53.9% using a standard catheter ($P < 0.001$). Although rates of successful cannulation were similar between the two groups (hydrophilic guidewire 83.8% *versus* standard catheter 84%) if instrument crossover occurred[10].

Development of the RX Biliary system in 1999 has led to increased control of the guidewire and exchange by the physician, decreased hand and wrist force used during contrast injection, and in return improved physician stress, efficiency, and speed. The changes in guidewire design and physician control of the wire can be expected to reduce ampullary trauma and lead to decreased complication rates and post-ERCP pancreatitis (PEP) in particular[11].

The studies to date have yielded conflicting results regarding the role of guidewire cannulation and prevention of PEP. Lella *et al*[12] conducted a prospective study with 400 patients randomized to either Group A with a guidewire used to access the pancreatic duct and endoscopic sphincterotomes, and Group B with a traditional catheter plus injection technique used. The rate of PEP was 0% in Group A *versus* 4.1% in Group B. One study which randomized patients to either primary contrast or guidewire-assisted cannulation (Jagwire; Boston Scientific Corporation) showed improvements in rates of cannulation, however, there was no reduction in the incidence of PEP (7.9% with guidewire and 6.2% with contrast). Increased attempts at cannulating the papilla demonstrated increased rates of PEP with > 10% after four or more attempts[9]. No difference in the rates of post-procedural pancreatitis after cannulation was shown by Katsinelos *et al*[10] (standard catheter 7.8% *versus* hydrophilic Jagwire 0.035-inch guidewire 5.4%).

Guidewire manipulation of the ampullary surface has been suggested to be less traumatic than contrast injection or forceful manipulation with a catheter. Double wire use is helpful in cannulation of the common bile duct. Pancreatic duct guidewire placement can be used to facilitate cannulation into the choledochus portion of the common bile duct by maintenance of orientation for the endoscopist[13-15].

A major advantage of the short-wire system is the potential for shorter procedure and fluoroscopy time. Papachristou *et al*[16] showed that using the V-system endoscope and accessories with a short hydrophilic wire (Glidewire; Boston Scientific Corporation) can lead to rapid and reliable device exchanges with only a 5% chance of wire loss. In some exchanges the authors used the so called "hydraulic technique". The hydraulic technique uses standard techniques to achieve access with the Glidewire and catheter until all available wire is inserted into the catheter. Then, water is flushed under pressure into the catheter, keeping the wire in place, while the catheter is removed by the endoscopist after confirmation of the wire position[16,17]. Over half of the instances of wire loss were either unrelated to the exchange or required minimal adjustment due to partial loss. All endoscopists regained wire access and one endoscopist was able to reduce his average number of guidewires used per case from two to one[16]. The use of continuous fluoroscopy was also avoided with maintenance of the Glidewire position. The technique described above can permit access to the pancreaticobiliary tree and allow stent insertion in complicated cases with less difficulty than standard methods. The ability of the V-scope to hold the Glidewire varied between exchanges regardless of endoscopic position, but still resulted in faster exchanges than a regular duodenoscope with no change in wire loss rates[16].

A prospective multicenter, randomized and controlled trial was conducted by Joyce *et al*[8] to compare the V-system (Olympus XTJF-140V2F) with the traditional duodenoscope and accessories. The V system scope elevator lever includes a V-shaped groove and an increased angle of articulation in comparison to the standard Olympus TJF-160. The V-system was found to have both reduced rates of guidewire adjustments and time needed to complete accessory exchanges over a guidewire when compared to the traditional system. Reduction in exchange time between the V-system and the conventional system was 19.4 s *versus* 31.7 s ($P < 0.001$)[8], whereas the need to reposition the guidewire was required less often with the V-system, 9.4% *versus* 35.7% ($P = 0.0005$)[8]. In contrast, the reduced procedure and fluoroscopy times were not found to be statistically significant[8]. Failure to secure the guidewire leading to loss of access occurred in 11% of cases[8]. Reasons for loss of access varied from unfamiliarity with the system to nuances with the use of the guidewire and the elevator.

The intraductal exchange technology offered by the Fusion system allows the guidewire to be detached from the catheter within the bile or pancreatic duct. When aggressive endoscopic management is necessary for drainage of pancreatic pseudocysts, this system allows for placement of a number of plastic stents with less effort than traditional methods[6]. There is elimination of both exchange outside the endoscope and multiple cannulations for reentry into the ducts or the pseudocyst cavity. Use of a second guidewire through a cystotome and the intracystic wire exchange technique secures access to the pseudocyst[6].

Preliminary data from a prospective randomized single-blinded trial with 46 patients that compared performance characteristics of the short-wire ERCP system (Fusion) and a standard long wire system (DASH), showed a trend towards shorter procedure times and shorter time to perform various ERCP maneuvers with the short-wire system[7]. A statistically significant reduction in stent insertion times were also observed during this study ($P = 0.001$).

Sai *et al*[18] used the Fusion system for placement of double plastic stents for the palliation of lower biliary obstruction associated with unresectable pancreatic cancer. Successful stenting was accomplished in 94% (15) of patients with two requiring balloon dilatation of the stricture. No complications related to stent insertion and retrieval occurred. Mean patency duration was 151.1 d.

Johlin *et al*[17] at the University of Iowa described the use of a standard catheter-type device in combination with a short 0.035-inch guidewire (240 cm in length). The authors used a 3-mL syringe and sterile water to perform hydraulic exchanges as described earlier. They documented that the entire hydraulic ERCP catheter

Table 2 Short-wire system potential advantages

Advantages
Reduced exchange times
Reduced stent insertion times
Maintenance of ductal access
Reduction of sedation and fluoroscopy time
Increased endoscopist control of cannulation
Locking of wire in position to increase stability
Decreased rates of post-ERCP pancreatitis
Decreased trauma when ampullary surface is manipulated
Reduced rates of wire adjustments
Aids in stricture access
Allows placements of multiple stents (Fusion system only)

Table 3 Short-wire system potential drawbacks

Drawbacks
Only preliminary studies have documented the potential benefit of the short-wire systems (all systems)
Hydraulic exchange technique not plausible with RX system
Decreased pushability with the open channel design of the RX system
Inability to flush channel for hydrophilic wire use (RX system)
Inability to use smaller than 0.035 inch or angled wires when channel is torn after first device exchange (unless device is preloaded) (RX system)
Deterioration of the device after multiple exchanges (RX, Fusion systems)
No easy method for insertion of pancreatic stents (all systems)
No reliable method of locking wire (V-system)
Looping of wire between the biopsy port and the external locking device (RX, Fusion systems)
Poor guidewire visibility (V-system)
Air and bile leakage causing increased soiling of operators (RX, Fusion systems)
Wires may suspend freely in air after being locked jeopardizing operators (all systems)
Loss of visibility due to decreased distention of the duodenum (RX, Fusion systems)

exchange took less than 30 s. Over the past 10 years this system was shown to save time, save money, maintain capacity to aspirate bile and pancreatic fluid and decrease contamination (if a short-wire is accidentally dropped it will not touch the floor). Tables 2 and 3 summarize the potential benefits and drawbacks of the use of short-wire systems.

DRAWBACKS

Although there are many benefits to the short-wire system, there have been some inefficiencies associated with them. One study showed that by using a dedicated short-wire monorail catheter with an accessory system (the RX system), slower exchange times by an average of 4 s were observed when compared to standard accessories. The RX system is not amenable to the hydraulic exchange technique[16]. In contrast to other studies, one prospective study showed that the time required for primary selective common bile duct cannulation was increased in the hydrophilic guidewire group at 4.48 ± 0.32 min *versus* the standard catheter group at 3.53 ± 0.32 min[10]. Other potential problems: decreased pushability due to the open channel design (RX), inability to flush the channel to facilitate use with a hydrophilic wire (RX), inability to use smaller than 0.035 inch or angled wires after the channel is torn with the first device exchange (unless the device is preloaded) (RX), deterioration of the device after multiple exchanges (RX, Fusion), not being able to insert pancreatic stents easily (all), no reliable locking of the wire (V), looping of the wire between the biopsy port and the external locking device (RX, Fusion), poor guidewire visibility (V), air and bile leakages which may lead to soiling of the operator and loss of visibility due to decreased distention of the duodenum.

SAFETY

The safety of short-wire systems has not been addressed exclusively in any published studies. Damage to the guidewire may occur with external locking of the wires. In addition, the proximal end of the shortest wire freely suspends in air after being locked and can present a risk to the operator, assistant, or patient[4]. Antileak caps should consistently retain air and bile preventing any spray of secretions but failure of this feature may lead to adverse outcomes.

When using the internal locking endoscopes, inappropriate locking of the wire leading to access loss may require repeated cannulation of the guidewire. As devices are introduced when using the internal locking endoscopes it is important that the V-shaped elevator is engaged properly[4]. If there is difficulty, it is important to note if there is damage to the tip of the device or catching of the guidewire in the space between the V-groove and the working channel[4].

Guidewire insertion into the bile duct improves the safety margin of a sphincterotomy by ensuring the incision of the biliary sphincter as intended. As mentioned before, this allows for repeated cannulation decreasing any risk of papillary injury if the papillotome becomes dislodged[12].

A search of the Maude database for all three short-wire system manufacturers was carried out and only adverse events regarding the Wallstent RX Biliary (Boston Scientific, Galway, Ireland) were listed. Adverse events included distal biliary duct stent occlusion 7 d after placement, hyperplasia at the site of the distal common bile duct stent 10 mo after placement during a stricture revision procedure, and a stent was found to have "foreshortened and proximally migrated into the bile duct" 1 year after placement. These complications were most likely related to the Wallstent design rather than to the use of the RX system.

COST

Using the short hydrophilic 0.035-inch biliary guidewire as the sole guidewire during a procedure decreases the need for a second wire, which may minimize cost during ERCP[16].

CONCLUSION

The practice of using ERCP short guidewire systems was developed to improve procedural outcomes. Although the use of traditional guidewires is vast amongst endoscopists, exposure to these systems may aid physicians to reduce exchange times, increase endoscopist control, reduce sedation exposure, reduce fluoroscopy time, increase stability with integrated lock systems and decrease rates of trauma during the procedure.

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Colorectal cancer surveillance in inflammatory bowel disease: The search continues

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Abstract

Patients with inflammatory bowel disease (IBD) are at increased risk for colorectal cancer (CRC). Risk factors for the development of CRC in the setting of IBD include disease duration, anatomic extent of disease, age at time of diagnosis, severity of inflammation, family history of colon cancer, and concomitant primary sclerosing cholangitis. The current surveillance strategy of surveillance colonoscopy with multiple random biopsies most likely reduces morbidity and mortality associated with IBD-related CRC. Unfortunately, surveillance colonoscopy also has severe limitations including high cost, sampling error at time of biopsy, and interobserver disagreement in histologically grading dysplasia. Furthermore, once dysplasia is detected there is disagreement about its management. Advances in endoscopic imaging techniques are already underway, and may potentially aid in dysplasia detection and improve overall surveillance outcomes. Management of dysplasia depends predominantly on the degree and focality of dysplasia, with the mainstay of management involving either proctocolectomy or continued colonoscopic surveillance. Lastly, continued research into additional chemopreventive agents may increase our arsenal in attempting to reduce the incidence of IBD-associated CRC.

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INTRODUCTION

Patients with both ulcerative colitis (UC) and Crohn's disease (CD) are at an increased risk of developing colorectal cancer (CRC). It is believed that this increased risk is a result of persistent inflammation of the colon. The exact mechanism as to how chronic inflammation results in carcinogenesis is unclear, but it is postulated that the same genetic mutations that result in sporadic CRC are also responsible for its development in inflammatory bowel disease (IBD). Surveillance guidelines employing colonoscopy as a tool for screening this high-risk population are available. Unfortunately, the guidelines have their limitations. Additionally, no consensus exists regarding the management of low-grade dysplasia (LGD) in the setting of IBD. This article will review the CRC risk factors in IBD, current surveillance guidelines, management of dysplasia in IBD, and lastly chemoprevention.

EPIDEMIOLOGY OF CRC IN PATIENTS WITH IBD

It has been known for nearly a century that UC is associated with an increased risk of CRC[1,2]. However, the association between CRC and CD has only recently been recognized[3-5]. The mean duration from the time of diagnosis of UC to the development of CRC is 17 years, with the mean age at diagnosis of CRC being 51 years in men and 54 years in women[6]. In a large meta-analysis

involving 54478 patients and 116 studies, Eaden *et al*[7] calculated the cumulative risk of CRC in UC at 8.3% at 20 years and 18.4% at 30 years. These results, however, have not been replicated, with more recent studies showing a lower annual incidence (0.06%-0.2%) of CRC in patients with UC[8,9]. Interestingly, two separate population-based studies from Denmark and the Mayo Clinic found no increased risk between CRC and UC when compared to the general population[10,11]. The differences among these studies have been postulated to be the result of improved medical therapies with unforeseen chemoprevention, increased colonoscopic surveillance, and improved surgical treatments.

Although there is less data regarding the risk of CRC in CD, it is well established that a similar association exists to that of UC[3-5]. However, the development of dysplasia in CD can involve both the small bowel as well as the colon. In a population-based study from the Mayo Clinic, only a slight increased risk was found between CD and CRC, which is in stark contrast to a 40-fold increased risk of developing small bowel malignancy[10]. Interestingly, an earlier large population-based study from Sweden reported a relative risk of 5.6 for the development of CRC in patients with Crohn's colitis[3].

PATHOGENESIS OF CRC IN PATIENTS WITH IBD

The exact mechanism by which chronic inflammation results in carcinogenesis is unclear. Persistent inflammation is believed to result in increased cell proliferation as well as oxidative stress, and ultimately the development of dysplasia[12-15]. It is postulated that similar genetic mutations that result in sporadic CRC in the general population are also responsible for its development in IBD, but the sequence of events and frequency are altered[16,17]. These events include microsatellite instability, inhibition of regulatory genes *via* hypermethylation of the promoter regions, and loss of adenomatous polyposis coli (APC), p53, and K-ras tumor suppressor function[12,18]. In sporadic CRC among the general population, loss of APC function generally occurs early and is frequent, whereas p53 mutations occur late and are less frequent. In contrast, loss of APC function generally occurs late and is infrequent, whereas p53 mutations occur early and are more frequent in IBD-associated CRC[18].

RISK FACTORS FOR CRC IN PATIENTS WITH IBD

Multiple risk factors for the development of CRC in the setting of IBD have been identified. It is well established that greater disease duration and anatomic extent of colitis are important risk factors[19]. Generally, patients with pancolitis develop CRC a decade prior to patients with left-sided colitis. Of note, disease extent is defined as the furthest extent of inflammation, either microscopically or macroscopically[20]. Recent studies have also demonstrated that the degree of colonic inflammation is associated with an increased risk of dysplasia and CRC. This was first introduced by the group at St. Mark's Hospital[21], and further confirmed by two recent studies[22,23]. Additionally, other markers of inflammation, including the presence of pseudopolyps, strictures, and backwash ileitis have been found to be independently associated with an increased risk of developing CRC[24-26].

Independent of a family history of IBD, a family history of sporadic CRC imparts a two-fold higher risk of CRC in IBD patients[27,28]. Also, IBD diagnosis at an earlier age increases the risk of CRC, independent of disease duration[19]. In fact, a Swedish population-based cohort of 3117 patients diagnosed with UC between 1922 and 1983, found a four-fold increase in CRC in patients diagnosed with UC prior to the age of 15 compared with those diagnosed between 15-29 years of age[19]. Lastly, the concomitant presence of primary sclerosing cholangitis (PSC) confers a significantly increased risk of CRC in patients with UC[29]. Among patients with PSC, Kornfeld *et al*[30] calculated a cumulative risk of developing CRC of 33% at 20 years and 40% at 30 years after the diagnosis of UC.

DEVELOPMENT OF DYSPLASIA IN IBD

Unlike sporadic CRC in the general population, the development of carcinogenesis in IBD does not always follow a sequential progression from LGD, to high-grade dysplasia (HGD), and ultimately to cancer[18]. In fact, Ullman *et al*[31] revealed that cancer can arise in patients with no prior dysplasia, or without first progressing from LGD to HGD. Additionally, they found that patients undergoing colectomy for flat LGD were found to have much more advanced pathology on surgical specimens. Also, CRC arising in the background of IBD is often multifocal and more aggressive than sporadic CRC[32-34]. This unpredictable sequence of events coupled with its more aggressive nature highlights the importance of increased vigilance and surveillance for CRC in this high-risk population.

Dysplasia is defined as the unequivocal neoplastic alteration of the epithelium without invasion into the lamina propria[35]. Macroscopically, dysplastic lesions in the setting of colitis can range from flat lesions (not endoscopically visible) to plaque-like lesions, to raised localized or multifocal lesions[36,37]. Raised, endoscopically visible lesions noted within areas of active colitis are referred to as dysplasia-associated lesions or masses (DALMs)[36]. These are further categorized into adenoma-like lesions and non-adenoma-like lesions, which ultimately results in different management implications[38]. Microscopically, per the IBD Dysplasia Morphology Study Group, dysplasia is divided into three categories: (1) negative for dysplasia; (2) indefinite for dysplasia; and (3) positive for dysplasia, which is further divided into LGD and HGD[35]. Unfortunately, inter-observer agreement and intra-observer reliability among

pathologists is variable in the diagnosis of dysplasia, especially when diagnosing indeterminate and LGD[39,40]. Therefore, it is traditionally recommended that any diagnosis of dysplasia be confirmed by a separate GI pathologist[41].

CRC SURVEILLANCE IN IBD

Recommendations for CRC screening/surveillance in IBD is aimed at early detection and mortality reduction from CRC. Although a multitude of studies exist demonstrating the need for secondary prevention[42-45], a 2004 Cochrane Database review did not show "clear evidence that surveillance colonoscopy prolonged survival." However, this analysis indirectly revealed a reduced mortality risk in IBD-associated CRC[46].

The most recent surveillance strategy, published in 2005 by the Crohn's and Colitis Foundation of America (CCFA), is based on expert consensus of past surveillance guidelines as well as more recent studies on IBD-associated dysplasia[41]. Per the consensus guidelines, screening colonoscopies should begin 8-10 years after the onset of IBD symptoms in patients with UC pancolitis/left-sided colitis and Crohn's colitis involving at least one third of the colon. The exception to this rule is that patients with concomitant PSC begin yearly surveillance colonoscopies at the time PSC is diagnosed. The timing of follow-up surveillance colonoscopies depends on the presence of dysplasia. If the initial colonoscopy in UC pancolitis/left-sided colitis (and Crohn's colitis) is negative for dysplasia, repeat surveillance colonoscopy should be performed in 1-2 years. Once a patient has two negative surveillance colonoscopies, further surveillance colonoscopies should be performed every 1-3 years. After 20 years of disease duration, surveillance colonoscopies should be repeated every 1-2 years. Management of dysplasia is described below. The recommended surveillance biopsies are four-quadrant biopsies every 10 cm with jumbo forceps. Of note, patients with only proctosigmoiditis of < 35 cm disease extent should follow the standard CRC screening guidelines for the general population, as they are not at an increased risk of developing CRC[41].

MANAGEMENT OF DYSPLASIA

All dysplastic and indefinite-for-dysplasia biopsies should be confirmed by an expert GI pathologist. Once confirmed, patients with HGD or multifocal flat LGD should be referred for prophylactic total proctocolectomy. Patients with indefinite dysplasia should undergo a more aggressive surveillance plan, with repeat surveillance in 3-6 mo. Controversy exists regarding the management of unifocal flat LGD, as studies have calculated a wide progression rate of 2%-50% from LGD to HGD[31,47-49]. Therefore, it is imperative that an open dialogue regarding the risks and benefits of both surgical and intense colonoscopic surveillance (repeat surveillance colonoscopies every 3-6 mo) be discussed with all patients found to have unifocal flat LGD. If intense surveillance is chosen by the patient, the CCFA consensus strongly recommends a proctocolectomy if multifocal flat LGD, repetitive flat LGD, or more advanced dysplasia is found during subsequent surveillance colonoscopies[41].

All non-adenoma-like DALMs should be referred for a complete proctocolectomy, as up to 50% of these patients can have cancer at the time of surgery[35,50]. Per the CCFA consensus guidelines, adenoma-like DALMs should be resected in their entirety, with the base and surrounding mucosa biopsied separately. Patients should be referred for surgery if the base or surrounding mucosa contains dysplasia. However, if no dysplasia is found in the base or surrounding mucosa, a repeat colonoscopy should be performed in 6 mo[41]. A summary of currently accepted algorithm for surveillance of CRC and management of dysplasia is presented in Figure 1.

Lastly, special attention should be applied to all colonic strictures in IBD. In UC patients, strictures should prompt surgical referral, given the high association with underlying malignancy[41]. Although the risk of malignancy is not as high in Crohn's-related colonic strictures, efforts should be made to visualize the colon proximal to the stricture, since an increased risk of CRC does exist. In fact, a 6.8% frequency rate of colon cancer after 20 years disease duration was found among a review of 175 Crohn's colon strictures[51]. Surgical resection should therefore be considered in longstanding CD, or if the colon proximal to the stricture cannot be visualized[41,51].

Although guidelines are available for CRC surveillance in IBD, multiple limitations exist. Per the current four-quadrant biopsies every 10 cm guideline, only less than 1% of the entire mucosal surface of the colon is sampled, leaving a very high sampling error[18]. Additionally, pathologists do not universally agree on the diagnosis of low-grade or indefinite-for-dysplasia, especially in a field of active inflammation[39,40]. Lastly, the management of flat LGD is often debated, specifically whether to proceed directly to surgery or follow a more aggressive colonoscopic surveillance plan.

CHEMOPREVENTION

The use of pharmacotherapy as a potential chemopreventive measure to reduce the risk of developing CRC has been studied extensively. The most widely studied agent for chemoprevention is 5-aminosalicylate (5-ASA). Unfortunately, no prospective studies evaluating 5-ASA as a chemopreventive agent exist, and the data is otherwise conflicting. The most compelling data from a meta-analysis of nine observational studies demonstrated a reduced risk of developing CRC or dysplasia with 5-ASA (OR = 0.51, 95% CI 0.38-0.69)[52]. This is in contrast to a retrospective case-controlled study of 25 patients with IBD and CRC, which demonstrated no association between 5-ASA

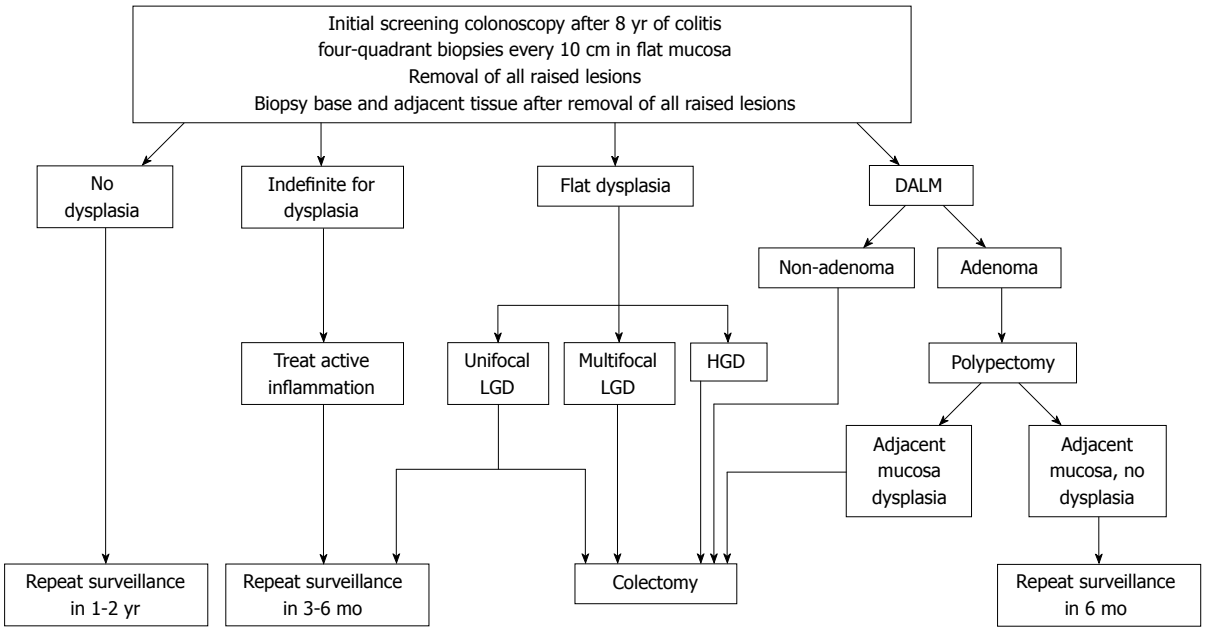

Figure 1 Algorithm for screening/surveillance for CRC in IBD and management of dysplasia. CRC: Colorectal cancer; IBD: Inflammatory bowel disease; LGD: Low grade dysplasia; HGD: High grade dysplasia; DALM: Dysplasia-associated lesions or masses.

use and reduction in CRC risk[53]. Nonetheless, with its relatively low side-effect profile, 5-ASA appears to be protective against CRC in IBD.

Additional agents studied for their chemopreventive effects include ursodeoxycholic acid (UDCA), corticosteroids, NSAIDs, folate, statins, calcium, and immunomodulators[18]. Two separate studies have demonstrated that the use of UDCA in patients with concomitant PSC and UC lowers the incidence of developing dysplasia or CRC (OR = 0.18 and 0.26, respectively)[54,55]. Thus, the use of UDCA for chemoprevention in patients with both UC and PSC is to be encouraged. However, the role of UDCA as a chemopreventive agent in UC without PSC is unknown. Currently, insufficient data or inadequate evidence precludes the use of folate, corticosteroids, NSAIDs, calcium, statins, and immunomodulators as chemoprotective agents against CRC[24,56-59].

ADVANCES IN DYSPLASIA DETECTION

As a result of the difficulty in identifying flat dysplastic lesions, the use of magnification chromoendoscopy was introduced. Magnification chromoendoscopy enhances mucosal contrast *via* the application of stains/dyes. Ideally, this would highlight mucosal changes that would otherwise not be seen by traditional white light endoscopy[60]. Methylene blue and indigo carmine are the most commonly studied dyes in UC surveillance[61,62]. In addition to stains/dyes, advances in imaging technique, including narrow band imaging and confocal laser endomicroscopy may further enhance the ability to target abnormal areas of the colonic mucosa, potentially even at the subcellular level. Currently, data on these new techniques are limited, and are not included, at this time, in surveillance guidelines[63].

CONCLUSION

The risk of CRC in long-standing UC and CD has resulted in the adoption of surveillance strategies with the goals of reducing morbidity and mortality associated with IBD-related CRC. Risk factors for the development of CRC in the setting of IBD include disease duration, anatomic extent of disease, age at time of diagnosis, severity of inflammation, family history of colon cancer, and concomitant PSC. Although guidelines currently exist, limitations of these guidelines, including sampling error at time of biopsy, interobserver disagreement in histologically grading dysplasia, and disagreement about the management of LGD indicate the need for continued research into the molecular pathogenesis of IBD-associated CRC, with the hope of identifying targets for prevention. Advances in endoscopic imaging techniques are already underway, and may potentially aid in dysplasia detection and improve overall surveillance outcomes. Management of dysplasia depends predominantly on the degree and focality of dysplasia, with the mainstay of management involving either proctocolectomy or continued colonoscopic surveillance. Lastly, continued research into additional chemopreventive agents may increase our arsenal in attempting to reduce the incidence of IBD-associated CRC.

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Evaluation and management of patients with refractory ascites

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Abstract

Some patients with ascites due to liver cirrhosis become no longer responsive to diuretics. Once other causes of ascites such as portal vein thrombosis, malignancy or infection and non-compliance with medications and low sodium diet have been excluded, the diagnosis of refractory ascites can be made based on strict criteria. Patients with refractory ascites have very poor prognosis and therefore referral for consideration for liver transplantation should be initiated. Search for reversible components of the underlying liver pathology should be undertaken and targeted therapy, when available, should be considered. Currently, serial large volume paracentesis (LVP) and transjugular intrahepatic portasystemic stent-shunt (TIPS) are the two mainstay treatment options for refractory ascites. Other treatment options are available but not widely used either because they carry high morbidity and mortality (most surgical options) rates, or are new interventions that have shown promise but still need further evaluation. In this comprehensive review, we describe the evaluation and management of patients with refractory ascites from the prospective of the practicing physician.

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Key words: Refractory ascites; Aquaretics; Albumin infusion; Transjugular intrahepatic portosystemic stent-shunt; Large volume paracentesis

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INTRODUCTION

Ascites means pathological fluid accumulation within the abdominal cavity. The word "ascites" itself is derived from the Greek word "askos," which means a bag or sack[1]. Cirrhosis accounts for over 75% of patients who present with ascites[2]. Ascites is the most common of the three major complications of cirrhosis; the other complications are hepatic encephalopathy and variceal hemorrhage[3]. Approximately 50%-60% of patients with compensated cirrhosis will develop ascites during 10 years of observation[3,4].

Patients with ascites can be divided into the following categories based on their response to treatment. (1) Less than 10% have natural sodium excretion (i.e. without diuretics) more than 78 meq/d. These patients, have relatively preserved liver functions and will respond to dietary salt restriction [88 meq (2000 mg) per day] alone[5]. (2) As liver function deteriorates, patients excrete less sodium in the urine and sodium restriction alone is no longer enough to create a negative sodium balance and control ascites[6]. Most patients will need diuretics combined with sodium-restricted diet[7]. This regimen is effective in about 90% of the patients[8]. Over time, up to 20% of patients that were initially diuretic-responsive will become diuretic-resistant[9]. (3) 5%-10% of patients never respond to this regimen and have refractory ascites[4,10].

Development of ascites is associated with a poor quality of life, increased risks of infections and renal failure, and a poor long-term outcome[11]. Furthermore, patients with refractory ascites have worse prognosis and shortened survival.

Cirrhotic patients who develop ascites have a probability of survival of 85% at 1 year and 56% at 5 years without liver transplantation[12]. In patients who

become resistant to diuretic therapy, the prognosis decreases to 50% survival at 2 years[13].

Patients with refractory ascites have lower sodium excretion compared to sensitive patients. It has been shown that patients with ascites and urinary sodium excretion below 10 meq/d had a mean survival rate of 5-6 mo compared to > 2 years in those with ascites and a higher rate of sodium excretion[14].

DEFINITIONS

For the correct diagnosis of true refractory ascites, the patient's condition should fulfill the following criteria[2,15].

Diuretic-resistant ascites

Failure of mobilization or the early recurrence of ascites which cannot be prevented because of a lack of response to sodium restriction and diuretic treatment is called diuretic-resistant ascites.

Diuretic-intractable ascites

Failure of mobilization or the early recurrence of ascites which cannot be prevented because of the development of diuretic-induced complications that prevent the use of an effective diuretic dosage is called diuretic-intractable ascites.

Treatment duration

Patients must be on intensive diuretic therapy (spironolactone 400 mg/d and furosemide 160 mg/d) for at least 1 wk and on a salt-restricted diet of less than 90 mmol/d.

Lack of response

Mean weight loss of less than 0.8 kg over 4 d and urinary sodium output less than the sodium intake.

Early ascites recurrence

There is an reappearance of grade 2 or 3 ascites (clinically detectable) within 4 wk of initial mobilization. However, it is important to notice that in patients with severe peripheral edema, reaccumulation of ascites within 2-3 d of paracentesis must not be considered as early ascites recurrence because it represents a shift of interstitial fluid to the intraperitoneal space[16].

Diuretic-induced complications

Diuretic-induced hepatic encephalopathy is the development of encephalopathy in the absence of any other precipitating factor. Diuretic-induced renal impairment is indicated by an increase of serum creatinine by > 100% to a value of > 2 mg/dL in patients with ascites otherwise responding to treatment. Diuretic-induced hyponatremia is defined as a decrease of serum sodium by > 10 mEq/L to a serum sodium of < 125 mEq/L. Diuretic-induced hypo- or hyperkalemia is defined as a change in serum potassium to < 3 mEq/L or > 6 mEq/L despite appropriate measures.

In addition to this, we should exclude dietary non-compliance (patient taking excess sodium in diet) and exclude the use of nonsteroidal antiinflammatory drugs (NSAIDs), which can induce renal vasoconstriction and diminish diuretic responsiveness[17,18].

EVALUATION OF PATIENT WITH REFRACTORY ASCITES

The aim of the work-up is to confirm the diagnosis and exclude other conditions that can be misdiagnosed as refractory ascites.

Exclude causes of ascites other than cirrhosis that are not responsive to diuretic therapy. This includes malignant ascites due to peritoneal carcinomatosis (but not due to massive hepatic metastasis)[19] and nephrogenic ascites, which develop in patients with end-stage renal disease[20]. This is important because about 5% of patients with ascites have more than one underlying etiology (mixed ascites)[21]; for example, a patient may have cirrhosis in addition to peritoneal carcinomatosis and be misdiagnosed as having true refractory ascites. The importance of this is that lines of therapy are different.

Patient should have ultrasound with portal vein Doppler and serum alpha fetoprotein level to exclude the presence of hepatocellular carcinoma or portal vein thrombosis[4], because these conditions are associated with lack of response to diuretics in patients with cirrhosis, while true refractory ascites represents actual progression of the liver disease[3] (discussed below).

Confirm compliance to dietary sodium restriction because patient may not be responding to diuretics simply because of the lack of dietary compliance. Therefore, the diagnosis of refractory ascites is not complete until it is proven that the patient has low urinary sodium excretion on the diuretic doses mentioned before[17]. This can be done through the following.

Twenty-four-hour urinary sodium: Patients who gain weight despite excreting more than 78 meq sodium per day are not compliant with the diet. The value of 78 meq sodium per day is derived from the difference between sodium intake (2 gm/d = 88 mEq) and non-urinary loss (10 mEq/d)[22]. The drawback of the 24-h urinary collections is that they are labor-intensive for patients and staff alike. Verbal and written instructions should be given to the patient in order to assure accurate collection. Completeness of collection can be assessed by measurement of urinary creatinine. Urinary creatinine excretion per day should be more than 15 mg of creatinine per kg of body weight for men and more than 10 mg/kg for women[23]. Samples with less creatinine indicate incomplete collection that may affect the results. However, this may not be very accurate because patients with advanced cirrhosis have muscle wasting and therefore lower creatinine excretion in urine even with complete collection[24,25].

Sodium in spot urine specimen: Measuring sodium in a spot urine specimen should be easier and more

convenient for the patient but lack of accuracy is the problem as excretion of sodium is not uniform throughout the day. Random urinary sodium concentrations are of value when they are 0 mmol/L (meaning low sodium excretion and lack of diuretic response) or greater than 100 mmol/L (means either adequate response to diuretics or diet non compliance) but are not helpful when they are intermediate[22].

Random urinary Na/K ratio: Random urinary Na/K ratio may be as helpful as 24-h urinary sodium collection, with accuracy rates of 86% according to one study and 90% according to another. A ratio of more than 1 is equivalent to 24 h sodium more than 78 mmol Na/d. This test is easier for the patient as it does not involve collection of 24-h urine.

Furosemide-induced natriuresis: Furosemide-induced natriuresis is another alternative where a single intravenous 80-mg dose of furosemide is given and urinary sodium is measured in the next 8 h. Patients with diuretic-resistant ascites have sodium excretion less than 50 mEq/8 h[26,27]. Another advantage of this test is that it allows more rapid identification of diuretic-resistant patients without the need to follow them up for weeks with increasing doses of diuretics.

PATHOGENESIS OF ASCITES

Currently, the most widely accepted theory of ascites formation is the forward theory which is based on the peripheral arterial vasodilation hypothesis of renal dysfunction in cirrhosis[28]. According to this theory, the initial step is the development of sinusoidal portal hypertension[29,30]. This leads to systemic vasodilation and reduction in systemic vascular resistance, which is most evident in splanchnic blood vessels[31,32]. Portal hypertension causes vasodilation through increased release of local vasodilators such as nitric oxide (which seems to be the primary mediator[33]), glucagon[34], prostacyclins[35], vasoactive intestinal peptide, substance P and platelet activating factor[29]. Splanchnic vasodilation leads to a forward increase in filtration across splanchnic capillaries[36]. In patients with decompensated cirrhosis, the lymphatic system is not capable of returning back all the filtered fluid and causes accumulation of ascites fluid[37]. Besides this, systemic vasodilation causes systemic vascular underfilling, which stimulates the sodium-retaining neurohumoral mechanisms in order to refill the dilated vascular bed; these mechanisms include mainly the renin-angiotensin-aldosterone system, sympathetic nervous system, and antidiuretic hormone[38]. This leads to sodium retention, water retention (with dilutional hyonatremia) and renal vasoconstriction, which may later lead to hepatorenal syndrome[28]. This causes retention of more fluid, which is not effective in filling the systemic vascular bed because of the continuous leakage into the peritoneal cavity leading to more ascites formation[28]. These changes become more severe with the progression of liver disease, which is why the degree of sodium retention (measured as urinary sodium excretion)[14] and hyponatremia[39,40] correlate with worsening survival in cirrhotic patients.

Patients with more advanced cirrhosis have a marked degree of circulatory dysfunction and marked neurohumoral activation. This results in renal vasoconstriction and enhanced sodium reabsorption in the renal tubule and very low urinary excretion of sodium (even with high doses of diuretics), which is how refractory ascites develops[17]. Hepatorenal syndrome has a pathogenesis similar to that of ascites[41]; refractory ascites is considered a pre-hepatorenal state and actually refractory ascites is a usual manifestation of type 2-hepatorenal syndrome[4].

TREATMENT OF REFRACTORY ASCITES

The ideal treatment of ascites should be effective in mobilization of ascites and prevention of recurrence, should improves patient's quality of life and survival, and should be acting directly on one or more steps in the pathogenesis of ascites and not just the mechanical removal of the fluid[42].

Currently, the main lines of treatment for refractory ascites are serial large volume paracentesis (LVP), transjugular intrahepatic portasystemic stent-shunt (TIPS), liver transplantation and peritoneovenous shunt[22]. We will also discuss promising new therapies that are currently being evaluated.

LVP

LVP with administration of intravenous albumin represents the standard therapy for refractory ascites[43]. Several studies have shown its effectiveness and safety[44-46]. Beside rapid control of ascites, it may decrease the risk of variceal bleeding because it is associated with reductions in the hepatic venous pressure gradient and intravariceal pressure[47,48].

Frequency of LVP: Therapeutic paracentesis is a local therapy that does not modify the mechanisms that lead to ascites formation. Therefore ascites will always recur in patients with refractory ascites unless there is an improvement in liver disease, as in alcoholic liver disease when patients stop drinking, or after liver transplantation[49,50]. Two weeks are considered a reasonable interval between paracentesis sessions in patients with refractory ascites[22,51]. Less frequent sessions are needed in the patient with some sodium excretion and more frequent sessions are required in patients who are not compliant with dietary sodium restriction. The explanation requires knowing some details related to sodium balance in patients with ascites. The sodium concentration of ascitic fluid is approximately equivalent to that of plasma in these patients: 130 mmol/L. A 10-L paracentesis removes 1300 mmol (130 × 10). If the patient is adherent to the diet, he/she will consume 88 mmol sodium every day, and excrete 10 mmol/d in non-urinary loss and excrete nothing in the urine if there is no urinary sodium

excretion at all. Therefore, the net gain every day will be 78 mmol. Therefore, a 6-L paracentesis removes 10 d (780 mmol/78 mmol per day) of retained sodium, and a 10-L paracentesis removes approximately 17 d of retained sodium (1300 mmol/78 mmol per day = 16.7 d) in patients with no urinary sodium excretion[22].

Patients who are not compliant need education regarding their diet rather than more frequent LVP sessions. This is important because although the patients are no longer responding to diuretics, diet is still very important. One should not think about a more restricted diet as a solution for diuretic resistance, as it is not more effective and makes food less palatable therefore, malnutrition may result[52]. Fluid restriction is indicated in patients with ascites and serum sodium lower than 130 mEq/L[53].

At the time of LVP measurement of the white cell count with differential should be performed on the acidic fluid sample as a screening for spontaneous bacterial peritonitis even if the patient is asymptomatic, while if symptomatic, cultures should be added[50].

Most authors prefer total LVP than repeated LVP (removing 4-6 L daily until ascites completely disappears) because it is faster and can be done as an outpatient procedure; also, it is associated with lower incidences of complications that may be related to needle insertion and associated with no fluid leakage after paracentesis because no fluid stays in the abdominal cavity[38]. Another measure to reduce leakage is using the "Z" track where skin is penetrated perpendicularly, then the needle is advanced obliquely in subcutaneous tissue before the peritoneal cavity is punctured, so that the puncture site on the skin and the peritoneum are not overlying. Also asking the patient to recline for 2 h on the side opposite to the paracentesis site will prevent the leakage of ascitic fluid. If there is significant leakage that is not controlled with these measures, a suture or purse string may be inserted around the site of drainage[54].

Complication associated with LVP: It is considered a safe procedure associated with very low incidence of serious complications even in patients with coagulopathy[55,56]. The risk of developing a large hematoma is about 1% and the risk of hemoperitoneum or iatrogenic infection is only about 1 per 1000[57]. There is no evidence in clinical trials that transfusion of plasma or platelets before the procedure decreases the risk of bleeding[58]. However, one should avoid puncture of the visible dilated abdominal wall veins in order to avoid severe bleeding. Also, there is no coagulation profile cut-off value that paracentesis should be avoided beyond it. According to one study, patients tolerated the procedures with INR up to 8.7 and platelet counts as low as 19 000[59]. It may be that the only condition when the procedure should be avoided due to high bleeding risk is the presence of disseminated intravascular coagulation with clinically evident fibrinolysis[60].

One problem with repeated LVPs is ascitic fluid protein and complement depletion, which may predispose to ascitic fluid infections[61], in comparison with diuretic therapy, but this is of special concern in diuretic-sensitive patients while in refractory ascites, diuretics are no longer an option.

Another problem with large volume paracentesis is post-paracentesis circulatory dysfunction (PCD). Circulatory changes after LVP can be described as follows. (1) Immediately after paracentesis, there is an improvement in circulatory function in regard to increased cardiac output and suppression of the renin-angiotensin and sympathetic nervous systems. This effect is mostly due to mechanical factors that mainly increase venous return due to reduced intraabdominal pressure[62]. (2) After about 12 h, there are opposite hemodynamic changes, including a reduction in cardiac output to baseline values and marked activation of the renin-angiotensin and sympathetic nervous systems over the levels before paracentesis[63]. These changes are not spontaneously reversed as once plasma renin activity and plasma norepinephrine concentration increase, this elevation persists[38]. PCD has been defined as a 50% increase in plasma renin activity over baseline on the sixth day after treatment, up to a value greater than 4 ng/mL per hour[38,62,64]. Despite being asymptomatic, PCD adversely affects the clinical course of the disease with higher incidences of hyponatremia, and renal impairment. In patients who develop PCD, it is severity correlates inversely with patient survival[65]. Severity of the circulatory dysfunction correlates with the amount of fluid removed in paracentesis being most significant when it exceeds 5L[65].

A number of measures can be applied to prevent PCD.

Albumin infusion: Albumin infusion has been studied for the prevention of PCD[45,66]. Incidence of PCD following LVP reaches 80% when albumin is not used and albumin infusion reduces the incidence to 15%-20%[45]. However, some controversy still exists related to albumin infusion. The reasons behind this are: lack of direct survival advantage with albumin infusion[67]; albumin is very expensive and some studies state that albumin infusion inhibits synthesis of albumin[58] and stimulates albumin degradation inside the body[68,69]. Another reason for the controversy is that the circulatory changes that can follow LVP may not be related to a decreased intravascular volume due to rapid accumulation of ascitic fluid as was thought before[70], but it is actually due to accentuation of the arterial vasodilatation already present in these patients[38].

The current American Association for the Study of Liver Diseases (AASLD) guidelines state that post-paracentesis albumin infusion may not be necessary for a single paracentesis of less than 4-5 L. For LVP, an albumin infusion of 8-10 g per liter of fluid removed can be considered[22].

Albumin should be given once the session is completed[54]. Some authors recommend giving one half of the plasma expander immediately after the paracentesis and the other half 6 h later[65,71]. Others say this is unwarranted and converts an otherwise simple

outpatient procedure into an all-day clinic visit[67].

It has been suggested that reducing the flow rate of ascites extraction may help prevent PCD[72], however, this may need further evaluation before being applied in practice.

Other alternatives to albumin for prevention of PCD: Many trials were carried out to find less expensive alternatives to albumin therapy, however none is accepted currently to replace albumin. Some of the alternatives to albumin infusion include synthetic colloids, extracorporeal ultrafiltration and reinfusion, and vasoconstrictors.

(Ⅰ) Synthetic colloids. Studies comparing replacement of albumin with dextran 70 or polygeline showed no survival advantage[71] and PCD was much less common with albumin administration in patients where ⩾ 5 L of fluid were removed[65]. Saline also has been tried but without showing any survival advantage[73]. This is mostly related to the half-life of the colloid used, being highest with albumin (21 d), this may explain its effectiveness in prevention of PCD[74]. Some authors state that paracentesis of < 5 L can be followed by synthetic plasma expander and albumin is not required in this setting[54].

One important point is that hydroxyethyl starch can increase portal pressure as it fills Kupffer cells (lysosomal storage) and this may increase the risk of variceal bleeding[75].

(Ⅱ) Extracorporeal ultrafiltration and reinfusion. This procedure involves ultrafiltration of ascitic fluid and intraperitoneal or intravascular reperfusion[76]. Advantages compared to albumin are the reduced expenses and avoidance of depletion of complement lost with paracentesis[77]. The main problem reported is the development of disseminated intravascular coagulation (DIC) in some patients, and this may be why it is not approved for use now; however, one simplified method is suggested that limits the incidence of DIC[78]. One point is that only a few patients have been studied until now therefore, better evaluation with larger studies is needed.

(Ⅲ) Vasoconstrictors. Administration of vasoconstrictors may decrease the development of PCD and may prevent complications associated with a decrease in effective arterial blood volume as with the plasma volume expander albumin[64]. This may be due to the fact mentioned before, as the pathogenesis of PCD is accentuation of splanchnic vasodilation rather than depletion of intravascular volume.

Terlipressin: More than one study showed that terlipressin may be as effective as intravenous albumin and well tolerated in preventing paracentesis-induced circulatory dysfunction in patients with cirrhosis after therapeutic paracentesis[79-82]. One study recommended a dose of terlipressin (1 mg every 4 h for 48 h)[79], while another study suggested that a total dose of 3 mg terlipressin should be administered as an intravenous bolus of 1 mg terlipressin at the onset of paracentesis and then 8 and 16 h after the first bolus[80]. A problem with using terlipressin is that it requires hospital admission for a simple outpatient procedure, as it is given as intravenous injections for up to 48 h after the procedure.

Midodrine: One study carried out on 40 patients showed that midodrine may be as effective as albumin[83], while another one carried out on 24 patients showed that PCD developed in six patients of the midodrine group (60%) and in only four patients (31%) of the albumin group[64]. The dose given was 12.5 mg every 8 h post-paracentesis for 2 d. Being much cheaper than albumin and terlipressin and much easier to administer, midodrine may be worth more trials to assess its use instead of albumin.

Noradrenaline: Another study showed noradrenaline to be as effective as albumin in the prevention of PCD[84]. Noradrenaline was suggested as a less expensive alternative to albumin but no further studies were done to confirm this.

TIPS

TIPS is a side-to-side portacaval shunt by which an intrahepatic communication between the portal and the hepatic vein is created[85]. It is a non-surgical procedure performed under local anesthesia by an interventional radiologist. A catheter is advanced through the jugular vein into a hepatic vein and into a main branch of the portal vein. There an expandable stent is introduced connecting hepatic and portal systems, which allows shunting of blood from the high-pressure portal circulation (splanchnic and sinusoidal beds) to the low-pressure systemic circulation (hepatic vein)[42].

The mechanism by which TIPS helps control ascites is decompression of the portal circulation and reduction in the portacaval gradient and the portal venous pressure[38]. As mentioned before, portal hypertension is essential in ascites formation so that cirrhotic patients with portal venous pressure less than 12 mm Hg do not develop ascites[29,30], and ascites in these patients disappears if portal venous pressure drops below 12 mm Hg[86,87]. Another mechanism is that the blood volume pooled in the dilated splanchnic vascular bed is transferred to the systemic circulation through the shunt, therefore, it corrects the systemic vascular underfilling and causes a decrease in the renin-angiotensin-aldosterone system and thereby improves renal sodium excretion[42,88].

Several studies showed that TIPS is highly effective in controlling ascites[54]. According to these studies, ascites was controlled in 27%-92%[89,90], with 75% of cases showing complete resolution[91]. It takes about 1 -3 mo for ascites to resolve after TIPS procedure[38]. One important point is that diuretic therapy will still be required in about 95% of patients. The explanation is that TIPS produces partial resolution of ascites pathogenesis, so portal pressure and renin and aldosterone levels, although they are markedly reduced after TIPS, they are not back to normal as in healthy subjects[38].

In patients with cirrhosis, TIPS may have some advantages beside ascites control. Improvement in

renal function is seen in these patients in the form of increased urine volume, increased sodium excretion[91,92] and even a reduction in serum creatinine level which is a delayed effect seen after 6 mo according to one study[93]. Another advantage is the improvement in the nutritional status (in the form of an increase in dry weight and total body nitrogen)[88,94] and improvements in quality of life[93]; however, these effects (nutrition and quality of life) may be simply due to improved eating when ascites is controlled[54].

Complications associated with TIPS: (1) Technical complications. Estimated technical success rate is reported in the range of 93%-100%[90,91]. Procedure-related mortality is very low (1%-2%) according to one study[95], and it was due to hemoperitoneum, hemobilia, hemolysis, and sepsis. The procedure-related complication rate is around 9%, with intraperitoneal hemorrhage and acute renal failure (mostly due to contrast media) being the most frequent[42]. Complications also include those of sedation and arrhythmia if the catheter enters the right atrium or right ventricle[96], and transient right bundle branch block, which may be significant in patients already with left bundle branch block as it may lead to complete heart block. The liver capsule is frequently punctured (reported frequency around 33%[97]), especially if the liver is shrunken but intraperitoneal bleed only occurs in 1%-2% of the cases[98]. (2) Hepatic encephalopathy occurs in about 30% of patients after TIPS[99,100]. Factors associated with the development of encephalopathy that can be used for patient selection are increasing age, advanced liver failure, and a history of encephalopathy before TIPS insertion[101,102]. Encephalopathy usually becomes clinically apparent 2-3 wk after TIPS insertion[99]. According to one study[100], encephalopathy starts to develop about 10 d after insertion, and then begins to decline (as measured by the portosystemic encephalopathy index) at 6 mo, however, it remained significantly higher than the baseline values. A possible explanation for this decline may be shunt stenosis with time. Treatment is medical in most of the cases and consists of controlling any precipitating factor, lactulose and non-absorbable antibiotics (neomycin or rifaximin)[100]. In case of medical therapy failure, the TIPS can be occluded[103] or the diameter of the shunt can be narrowed in some types with a "wasp waist" constrictor[104]. (3) Shunt occlusion. These problems occur in 22% to 50 % of patients[91,105,106]. This occurs due to growth of collagenous fibrils and endothelial cells (pseudointima) inside the stent[107]. It can diffuse all over the whole length (type 1) or localized to the hepatic venous end (type 2); however, both have the same management[108]. The incidence of this complication increases with time, according to one study, all patients surviving more than 2 years had shunt stenosis[108]. Shunt stenosis or occlusion presents as a recurrence of portal hypertension or variceal bleeding[109]. TIPS patency should be followed up after insertion, however, the best strategy for follow-up is not yet defined. Methods used to monitor patency include venography (which is the best test but not usually used because is invasive and caries a high cost), but Doppler sonography is the most frequently used (although it is less sensitive than venography)[110]. Helical CT angiography also can be used; one study showed a 92% correlation with venography[111]. One recommended approach for surveillance is duplex ultrasonography every 3 mo and venography annually. Venography can be done earlier if shunt obstruction is suspected clinically[110]. Treatment is redilation of the shunt done by interventional radiology[105]. New stents covered with polytetrafluoroethylene (Goretex) showed lower rates of occlusion and stenosis[112,113]. Using antiplatelet therapy was evaluated in the prevention of shunt stenosis[114] and it showed some efficacy, but it is not used in practice because of the increased risk of bleeding, as those patients already have bleeding tendencies and thrombocytopenia. (4) Haemolysis occurs in about 10% of patients and it is believed to be due to direct mechanical trauma to the red blood cells when they pass through the metallic stent[115]. This may be why spontaneous resolution is seen in most patients after 8-12 wk with covering of the metallic stent by pseudointima[116]. In most patients, the anemia is mild with less than 2 g/dL reduction in hemoglobin level starting 1-2 wk after placement. Blood smears may show schistocytes in patients who develop severe anemia[115]. (5) Infection. According to one study, infection occurred weeks to months after placement and presented with fever, continuous bacteremia and presence of vegetations or thrombi in the stent. It was treated with intravenous antibiotics[117]. (6) Portosystemic myelopathy (PSM, also called shunt myelopathy) is a rare syndrome that includes spastic paraparesis with intact sensation occurring in patients with surgical portosytemic shunts and also described after TIPS placement[118,119]. A possible explanation is accumulation of ammoniacal substances (that bypass the liver through the stent), leading to loss of motor neurons in the spinal cord. According to one study[118] carried out on 212 patients, four patients (1.89%) had this progressive spastic paresis starting to appear between 5 wk and 5 mo after stent placement. (7) Deterioration of cardiac function. TIPS increases the cardiac preload, and hence it may precipitate heart failure in those with pre-existing heart disease[120]. Echocardiography is usually done before the procedure to exclude patients with subtle heart failure; usually, patients with ejection fraction less than 60 are excluded.

TIPS versus paracentesis

Several studies have compared TIPS to repeated LVP plus albumin, but the detailed discussion of these studies is beyond the scope of this article. However, the conclusion from these studies is: (1) TIPS controls ascites effectively and is associated with a lower rate of ascites recurrence[106,121-123]; (2) patients with ascites who undergo TIPS improve their nutritional status as mentioned before; (3) there is a higher incidence of side effects, mainly hepatic encephalopathy and shunt dysfunction in the

group treated with TIPS; and (4) there is no proven effect for TIPS on survival. In one study, TIPS had no effect on survival[123,124], while others have reported both reduced[106] as well as improved survival[106,121,122] compared with therapeutic paracentesis. For these reasons, we believe repeated LVP plus albumin should be considered the first-line therapy for refractory ascites, and TIPS should be used as a second line of management[15,125,126]. TIPS should be considered in appropriately selected patients who meet the following criteria.

Patients with very rapid recurrence of ascites (those who require paracentesis > 3 times/mo) and preserved liver function [bilirubin < 3 mg/dL, serum sodium level >130 mEq/L, Child-Pugh score < 12, model for end-stage liver disease (MELD) score < 18], aged < 70 years, without hepatic encephalopathy, central hepatocellular carcinoma, or cardiopulmonary disease[15,127].

SURGICAL OPTIONS

Peritoneo-venous shunt is a surgically inserted shunt that drains ascitic fluid from the peritoneal cavity into the internal jugular vein. It has limited indications because there is no survival advantage in addition to frequent complications including bacteremia, small bowel obstruction and volume overload leading to variceal bleeding[10]. The use of the peritoneo-venous shunt is limited to patients with refractory ascites who are not candidate for TIPS or liver transplantation, and has a lot of abdominal scars that makes frequent paracentesis unsafe[22,67]. One study described percutaneous placement of a peritoneovenous shunt by interventional radiology which may carry less complications than surgery however further studies are needed to confirm this[128].

A more simple method of peritoneovenous drainage was described, the sapheno-peritoneal anastomosis[129-131]. Advantages over the ordinary peritoneovenous shunt are simpler and less expensive and use a biological shunt instead of a prosthetic one. Also, one study described the possibility of doing the procedure under local anesthesia[129], which is an advantage in cirrhotic patients. Patients had reduction in admissions and paracentesis, however, no survival advantage was noted.

Portosystemic shunt works, similar to TIPS, through decompression of the portal circulation; however, mortality is higher ranging from 12% to 39% and encephalopathy rates are more than 50%[132].

One study described a technique of peritoneal-urinary drainage of the fluid using a surgically implanted pump[133].

MEASURES THAT MAY IMPROVE THE RESPONSE TO DIURETICS

Several medications have been suggested that attack certain step(s) in the pathogenesis of ascites.

Aquaretics

Aquaretics are vasopressin receptor antagonists that act on the distal tubule of the kidney so as to increase the excretion of solute-free water[15]. They are already approved for management of hyponatremia due to syndrome of inappropriate anti-diuretic hormone secretion (SIADH), and are being evaluated for management of hyponatremia in cirrhosis and in refractory ascites to be combined with diuretics to improve response[134].

Vasopressin receptors are V1a, V1b and V2. The oral forms of aquaretics (e.g. lixivaptan and satavaptan) are selective for V2 receptors, which mediate the antidiuretic response of vasopressin. While, the intravenous forms (conivaptan) works on V2 and V1a receptors and V1a mediates the vasoconstrictor response of vasopressin[135]. Although conivaptan is the only approved one right now (used in SIADH), it cannot be used in ascites as it may cause variceal bleeding when it blocks the vasoconstrictor effect of the anti diuretic hormone[136].

One study carried out in 110 patients with cirrhosis and ascites receiving satavaptan or placebo in addition to diuretics[137]. Those receiving satavaptan had significant decrease in abdominal girth and more weight reduction without significant side effects. However, because of the short follow-up duration in this study (14 d), more studies are needed to evaluate the use of this drug in patients with ascites before they are approved for use in practice.

Vasoconstrictors

Vasoconstrictors may theoretically improve the action of diuretics as they improve the systemic vasodilation, so as to reduce the antinaturetic factors described before[15]. They are already used in hepatorenal syndrome based on a similar mechanism of action[138].

Terlipressin: A potent vasoconstrictor approved for use in the acute control of variceal hemorrhage and hepatorenal syndrome. In one study in 15 patients with cirrhosis and ascites without hepatorenal syndrome[139], eight of them had refractory ascites and received terlipressin. This group had significant decreases in plasma norepinephrine and renin in addition to an increase in urinary sodium.

Octerotide: According to a case report[140], octreotide treatment improved renal function and diuretic response in two patients with refractory ascites. Octreotide administration has been associated with arterial splanchnic vasoconstriction, which is mediated by a reduction in glucagon secretion. In addition, octreotide inhibits the release of renin and aldosterone in both normal humans and cirrhotic patients, possibly through a direct effect on renin-producing cells and adrenals.

Midodrine: One study carried out in 39 cirrhotic patients[141] evaluated the effects of a 7-d treatment with midodrine. It showed a significant increase in mean arterial blood pressure and urine volume and decrease in plasma renin and aldosterone activity in those with ascites treated with midodrine.

One study evaluated the combination of midodrine

and octerotide[142], another one evaluated both drugs given with albumen[143].

Clonidine

Several studies evaluated the response of diuretics in cirrhotic patients with ascites[144-147]. It is a centrally acting α2-agonist, therefore, it decreases sympathetic over activity which increases renal sodium reabsorption and stimulates the renin-angiotensin-aldosterone system[148]. One study was carried out in 32 alcoholic cirrhotic patients with ascites to compare the effect of spironolactone, clonidine and the combination of both in control of ascites[147]. After 10 d of spironolactone and clonidine, patients had a significant decrease in plasma renin and aldosterone, decrease in body weight and increase in natriuresis without adverse effects.

Chronic albumin infusion

Some studies showed better diuretic response when combined with albumin; this is noticed as decreased recurrence and shorter hospital admissions[149-151]. One study showed improved survival in patients receiving chronic albumin infusion[152]. Because of cost and lack of definite survival benefit, albumin infusion is not routinely recommended in patients with ascites. Currently, the accepted indications of albumin infusion in liver patients with ascites are with LVP to prevent PCD (discussed before), patients with spontaneous bacterial peritonitis[153,154] and those with hepatorenal syndrome[155,156].

Splenic artery embolization

One case report described the successful control of refractory ascites with splenic artery embolization[157]. This was a 32-year-old female who developed refractory ascites due to portal vein thrombosis after liver transplantation due to Budd Chiari syndrome. With further evaluation, this can be an alternative for patients with refractory ascites who cannot tolerate TIPS or surgical shunts.

Mannitol

In one study[158], a dose of 100 mL 20% mannitol was given as infusion followed by the usual dose of diuretics taken by the patient. Increase in urine volume and urinary sodium was noticed. Therefore, mannitol may be used in refractory ascites to improve response to diuretics.

The measures described above target mainly mobilization of ascites. In addition, as part of comprehensive strategy of managing refractory ascites, one can aim to prevent other complications of cirrhosis and also improve liver function by ether treating the underlying liver disease or liver transplantation. (1) Prevention of other complication of cirrhosis as patients with ascites due to liver cirrhosis are liable to other complications[7]. This includes portal hypertensive bleeding (prevention using either prophylactic banding or propranolol[159]), spontaneous bacterial peritonitis (using prophylactic antibiotics in patients with acute variceal bleeding or ascitic fluid protein less than 1 gm/dL[67]) and hepatorenal syndrome (using albumin infusion in patients with spontaneous bacterial peritonitis[154] and using pentoxyfillin in patients with severe alcoholic hepatitis[160]). (2) Correction of liver function through either liver transplantation or treatment of the underlying liver pathology: some causes of liver cirrhosis have a reversible element; this is most evident in patients with alcoholic liver disease in which stopping alcohol consumption can lead to improvement of portal hypertension and ascites control[161,162]. There is also some evidence of similar improvement in patients with cirrhosis due to hepatitis B (treated with antiviral therapy)[163] and patients with autoimmune hepatitis treated with steroids or azathioprine[164,165].

LIVER TRANSPLANTATION

As mentioned above, patients with ascites have a poor long-term outcome and survival is shortened in those who become refractory to diuretic therapy. The 12-mo survival rate for patients with ascites refractory to medical therapy is only 25%[166]. The survival rate for liver transplantation is much higher[67]. Therefore, those who develop refractory ascites ideally should be on the transplantation list already.

After liver transplantation, portal hypertension reversed immediately and completely; however, ascites disappearance may take 3 to 6 mo[13]. The reason for this is not fully understood, but some studies showed that the systemic vasodilation and hyperdynamic circulation persist for months after transplantation[167,168].

Priority of receiving liver transplant is based upon the MELD score. Some authors suggested that it may not be accurate enough for all patients with ascites, particularly for those with persistent or refractory ascites, who may have a poor prognosis despite low MELD scores[7,15]. One possible explanation is that the MELD score includes serum bilirubin, serum creatinine, and the INR, and some patients with refractory ascites might have a near-normal serum creatinine (as a result of low endogenous production), despite a low glomerular filtration rate and this may affect the accuracy of MELD score in this setting[4].

Therefore, some studies suggested that addition of serum sodium to the MELD score may improve its accuracy[169-171], but one point is that serum sodium is a not as steady as other parameters of the MELD score; it can change rapidly with diuretics or fluid administration, so this change may not reflect an actual change in the prognosis[15]. Further evaluation is needed before modified MELD (with serum sodium added to it) replaces the current MELD score in allocation of liver transplantation.

CONCLUSION

Refractory ascites is a relatively common condition in patients with liver cirrhosis. Wrong diagnosis may occur sometimes therefore, certain criteria should be fulfilled with exclusion of dietary non-compliance, which can

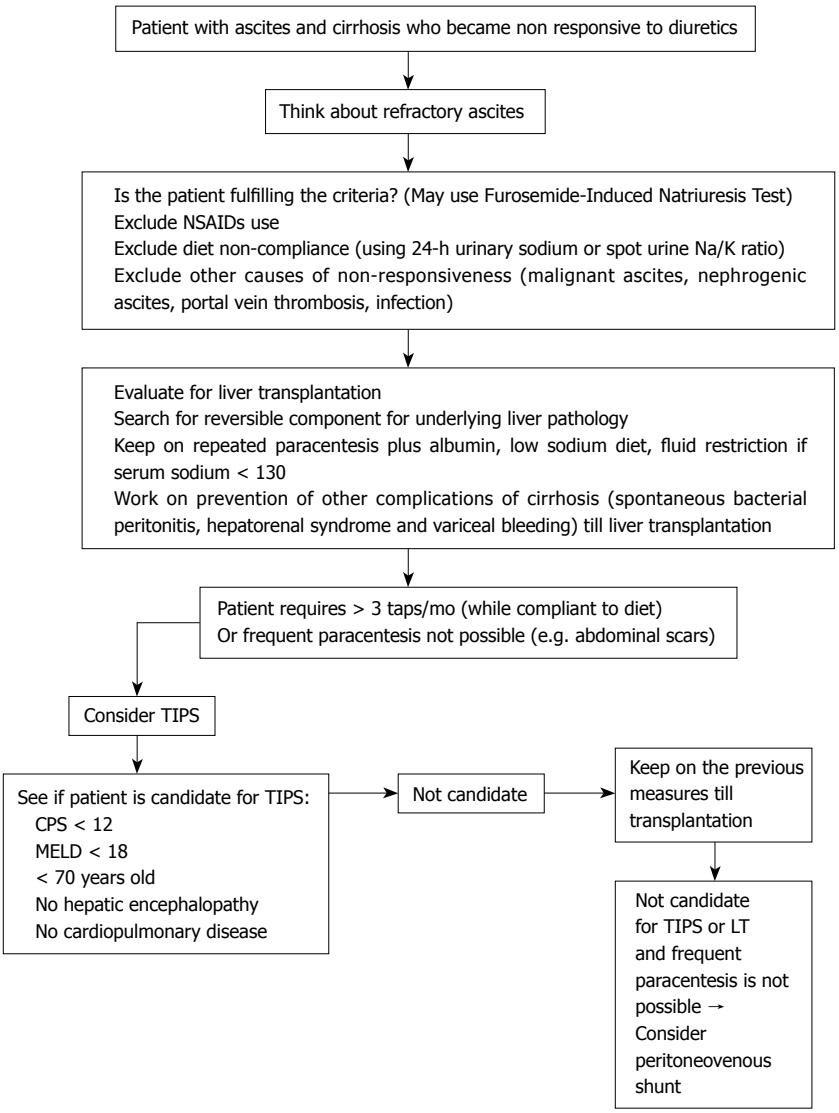

Figure 1 Suggested approach to the patient with refractory ascites. NSAIDs: Non-steroidal anti-inflammatory drugs; LT: Liver transplantation; CPS: Child-pugh score; MELD: Model for end-sage liver disease; TIPS: Transjugular intrahepatic portosystemic shunt.

be done through a variety of tests. Different treatment options are available, although definitive treatment is liver transplantation. An algorithmic approach to patients with refractory ascites is available (Figure 1). Other treatment options are not listed in the algorithm because they are still being evaluated and include mainly vasoconstrictor agents.

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Recent advances and remaining gaps in our knowledge of associations between gut microbiota and human health

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Abstract

The complex gut microbial flora harbored by individuals (microbiota) has long been proposed to contribute to intestinal health as well as disease. Pre- and probiotic products aimed at improving health by modifying microbiota composition have already become widely available and acceptance of these products appears to be on the rise. However, although required for the development of effective microbiota based interventions, our basic understanding of microbiota variation on a population level and its dynamics within individuals is still rudimentary. Powerful new parallel sequence technologies combined with other efficient molecular microbiota analysis methods now allow for comprehensive analysis of microbiota composition in large human populations. Recent findings in the field strongly suggest that microbiota contributes to the development of obesity, atopic diseases, inflammatory bowel diseases and intestinal cancers. Through the ongoing National Institutes of Health Roadmap 'Human Microbiome Project' and similar projects in other parts of the world, a large coordinated effort is currently underway to study how microbiota can impact human health. Translating findings from these studies into effective interventions that can improve health, possibly personalized based on an individuals existing microbiota, will be the task for the next decade(s).

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INTRODUCTION

In recent years, the commensal microbiota, including that of the small and large intestines, has received renewed research interest for potential associations with human health. The commensal microbiota consists of a diverse population of prokaryotic (eubacteria and archaea) as well as eukaryotic microbes that live synergistically within their human host. As early as the beginning of the 20th century, Metchnikoff proposed that putrefactive bacteria contribute to various disease processes and that modification of microbiota composition through consumption of viable microbes might help to improve health and longevity[1]. A variety of potential associations between gut microbiota composition/activities and health or disease have undergone scientific scrutiny. Evidence is mounting in support of an association between microbiota and diseases associated with failures in appropriate immune responses leading to excessive inflammation, such as atopic disease, inflammatory bowel disease and intestinal cancers[2,3,4]. During the next decade, findings from comprehensive microbiota studies currently underway can be expected to revolutionize the way we think about our microbial "friends and foes".

CONSUMPTION OF LIVE BACTERIA TO PROMOTE HEALTH

A wide variety of often milk-based fermented foods containing viable beneficial microbes, mostly lactic-acid bacteria and bifidobacteria, but also other bacteria

and fungi, have traditionally been part of the diet in many cultures. Various cultures share the belief that home-made products, such as yoghurt, curd, kefir, chal, kombucha *etc*, can help to maintain good health. Microbial cultures used for preparing these fermented products have sometimes been propagated for generations. They range from simple cultures that contain a few lactic-acid bacterial strains to complex consortia containing various bacteria and yeasts (kefir grains). More recently, commercial products claiming to contain beneficial bacteria that can establish residency in the gut (probiotics), fermentable substrates that enrich for beneficial bacteria (prebiotics), or mixtures of both (synbiotics), continue to expand their market share. Such products have been quite popular in Europe and Asia for a while but they are now also becoming more common in other parts of the world including the US. Although it is clear that there is significant potential for such products to help improve or maintain health, the research validating many of the current health claims is sparse. We discuss below some of the recent findings and future opportunities to advance this promising approach.

THE HUMAN/MICROBIOTA 'SUPERORGANISM'

It is well established that commensal microbial cells living in intimate contact with their human host far exceed the number of human cells. Bacteria belonging to a few phyla, particularly Firmicutes and Bacteroidetes, appear to dominate in most healthy individuals[5-7]. Estimates for the total number of bacterial species compromising the collective gut microbiome have recently been extended up to 40 000[8], but, due to the large amount of emerging sequence data, the bacterial species concept likely will soon undergo revision. Most gut microbiota research to date has focused on exploring the eubacterial community, but archaea (prokaryotes resembling bacteria but different in certain aspects of their chemical structure, such as the composition of their cell walls), viruses, fungi and other microbes can frequently be detected in intestinal contents[9-12].

The combined microbial gene pool, studied by metagenomic approaches, exceeds the complexity of the human genome, extending the metabolic abilities of the human/microbiota "supra- or superorganism"[3,13,14], which is the combined host/microbe consortium. Through its immense metabolic capabilities, the gut microbiota contributes to human physiology by transforming complex nutrients, such as dietary fiber or intestinal mucins that otherwise would be lost to the human host, into simple sugars, short-chain fatty acids and other nutrients that can be absorbed[5,15]. Furthermore, the microbiota produces some essential vitamins including vitamin K, vitamin B12 and folic acid, contributes to intestinal bile acid metabolism and recirculation, transforms potential carcinogens such as N-nitroso compounds (NOCs) and heterocyclic amines (HCAs) and activates bioactive compounds including phytoestrogens[16-18]. Differences in environmental factors, including diet, as well as hosts genetics are thought to contribute to microbiota diversity[18,19]. However, as genetically similar mice obtained from a dedicated breeding colony and fed the same amounts of the same defined diet develop striking differences in microbiota profiles[20], factors beyond our current comprehension or even random chance might contribute.

DISEASES ASSOCIATED WITH GUT MICROBIOTA DISTORTIONS

Distortions in any one of the microbiota functions or signaling pathways could potentially contribute to a wide range of diseases, including cardiovascular diseases (IBD) (bile-acid-associated regulation of serum cholesterol levels, chronic inflammation), diabetes (carbohydrate uptake and glycemic control), inflammatory diseases including atopic diseases, inflammatory bowel disease (inappropriate immune stimulation) and neoplastic diseases (carcinogen activation, chronic inflammation related hyperproliferation). Eloquent studies suggesting microbiota associations with obesity have recently received significant publicity[21-25] but other studies have refuted the existence of such an association[26]. Undoubtedly, the gut microbiota can contribute to differences in energy gain from fiber fermentation. The resulting small amounts of additional energy, if absorbed by the host, can over time, contribute to weight gain, and signaling from gut bacteria might contribute to fat storage. However, from a public health perspective, we might want to avoid shifting the focus away from a more direct path to avoid obesity: balance energy intake and output.

Changes in gut microbiota composition by probiotic supplementation of infant diets have been shown to reduce atopic disease[2,27]. Associations between the microbiota development in infants and health later in life have long been proposed[28,29]. Utilizing microarray technology to monitor microbiota, Palmer *et al*[30] recently reported changes in the microbiota composition in 14 infants during the first year of life, pointing to considerable temporal variation and distinct features in each infant.

IBD has been linked to microbiota composition in a variety of studies[3,7,31-36], and successful interventions using a pre- and/or probiotic approach have been reported. In addition to reports of differences in microbiota composition analyzed in fecal samples, the kinds and amounts of mucosa-adherent bacteria also seem to differ between cases with IBD and healthy controls[7,37-39].

Colorectal cancer (CRC) risk also has been proposed to be associated with microbiota composition through various mechanisms[4,40]. Pre- and or probiotics have reduced carcinogenesis in some but not all animal studies[41,42]. Dietary prevention of intestinal carcino-

genesis in APC^{Min} mice (mice that develop large numbers of intestinal tumors due to mutation in the adenomatous polyposis coli gene) was associated with correlated differences in overall microbiota profiles as well as with the presence of specific bacterial signatures[20]. Increases in the amounts of intraepithelial *Escherichia coli* (*E. coli*) in CRC patients have been suggested[43].

Interest has recently also been directed towards establishing a potential association between microbiota composition and both type 1 as well as type 2 diabetes mellitus. Brugman *et al*[44] showed that antibiotics affected type 1 diabetes mellitus incidence but, more importantly, that microbiota differed before the onset of disease in diabetes-prone rats that developed type 2 diabetes. Similar data have recently been reported in immune system-associated studies in non-obese diabetic (NOD)-mice[45]. Antibiotic-induced microbiota changes have also been shown to affect type 2 diabetes, but systemic effects likely contributed to this observation[46].

Current studies of associations between microbiota composition and disease suffer from a lack of understanding regarding the normal range of microbiota diversity on a population level. Furthermore, the presence of particular microbes or microbiota pattern has been studied almost exclusively in observational studies, in which differences in microbiota were evaluated between subjects suffering from the respective disease and normal controls. This study design does not allow us to distinguish if differences in microbiota composition are causing the disease or if they are simply a result of the changed gut environment in diseased subjects. Prospective studies evaluating microbiota composition in individuals before they develop disease will be required to attribute causality to potential associations between microbiota and disease. Because such microbiota studies would be expensive and time consuming, they should be designed as ancillary projects as part of larger cohort studies.

NEW OPPORTUNITIES TO STUDY GUT MICROBIOTA AND HEALTH

Powerful molecular microbiota analyses methods, including 16S rRNA sequencing through a massively parallel barcoded pyrosequencing approach, facilitate for the first time our ability to analyze microbiota in depth and in an efficient manner. Studies of gut microbiota interactions with metabolic phenotypes (so-called functional metagenomics) are now possible through the use of proton nuclear magnetic resonance (^{1}H NMR)-based profiling of fecal, urine or other extracts. Early results in this area that tried to correlated microbiota and probiotic supplementation-induced changes in its composition are promising[47,48].

Last year, the National Institutes of Health announced its roadmap Human Microbiome Project (HMP) with funding in excess of one hundred million US dollars, allocated to improve our understanding of associations between human health and microbiota at five major sites: nasal and oral cavities, gastrointestinal and urogenital tracts and skin[49] (http://nihroadmap.nih.gov/hmp/). Efforts to sequence the genomes of hundreds of human-associated microbes are currently underway and multiple projects that will explore potential associations with human health are currently being funded. European and Asian countries are undertaking similar endeavors and international efforts have been made to coordinate projects. It can be expected that the studies to determine the composition, activities and dynamics of the human microbiota and its overall genomic content, the human microbiome, will expand our ability to utilize microbiota for maintaining/improving health.

REMAINING GAPS AND CONCLUSIONS

Studies of microbiota composition have so far been limited to fairly small populations. We are clearly lacking an understanding of microbiota diversity on a population level and across various cultural and ethnic groups. Few studies have extensively investigated microbiota dynamics in adults; the causes for variations over time have not been well explored. The many interventions aimed at improving health parameters through microbiota modifications with pre-and probiotic supplements have often been short-term. Thus, effects of microbiota changes on long-term health are unknown. Furthermore, the types and concentration of pre- and probiotic supplements significantly vary from study to study, making firm conclusions difficult to draw.

To improve statistical power for defining disease-specific microbiota pattern, it is frequently necessary to combine results from various individuals into disease and control groups. However, it is crucial to recognize that inter-individual variations in microbiota composition may be so large and its statistical distribution so far from normal that combining individuals might not be appropriate. The true extent of microbiota variation will only be known after we have studied a sufficient number of individuals. Massive parallel sequencing technologies and the necessary bioinformatics tools to handle the resulting large datasets have been and continue to be adapted for human microbiota analysis[50,51].

To date, little effort has been made to standardize the microbiota analysis methodology used in human studies. Furthermore, the extent of the bias introduced by different sample collection, storage and analysis methods has only been superficially investigated. This makes it almost impossible to directly compare findings from different groups, limiting our ability to generalize findings.

Successfully correlating microbiota composition with disease risk, rather than correlating it with disease status only, will likely require large prospective epidemiological studies sufficiently powered to detect disease predicting microbiota differences, even with the predicted large inter- and intra-individual variation. Such findings could lead to future microbiota based preventions, which may have to be individualized based on the subjects' existing microbiota. It is also important to establish microbiota

changes that are caused by, but are not causally associated with, disease progression. Such knowledge might facilitate the development of efficient microbiota-based screening tests (IBD, CHC *etc*). We have all the reasons to be optimistic that, based on new findings, expected through the current large multi-national efforts to better understand microbiota, we will finally be able to 'domesticate' our own complex gut microbiota as a means for improving health.

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ORIGINAL ARTICLES

Prevalence of clonorchiasis in patients with gastrointestinal disease: A Korean nationwide multicenter survey

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Abstract

AIM: To investigate prevalence of *Clonorchis sinensis* in patients with gastrointestinal symptoms, and the relation of the infection to hepatobiliary diseases in 26 hospitals in Korea.

METHODS: Consecutive patients who had been admitted to the Division of Gastroenterology with gastrointestinal symptoms were enrolled from March to April 2005. Of those who had been diagnosed with clonorchiasis, epidemiology and correlation between infection and hepatobiliary diseases were surveyed by questionnaire.

RESULTS: Of 3080 patients with gastrointestinal diseases, 396 (12.9%) had clonorchiasis and 1140 patients (37.2%) had a history of eating raw freshwater fish. Of those with a history of raw freshwater fish ingestion, 238 (20.9%) patients had clonorchiasis. Cholangiocarcinoma was more prevalent in *C. sinensis*-infected patients than non-infected patients [34/396 (8.6%) *vs* 145/2684 (5.4%), $P = 0.015$]. Cholangiocarcinoma and clonorchiasis showed statistically significant positive cross-relation ($P = 0.008$). Choledocholithiasis, cholecystolithiasis, cholangitis, hepatocellular carcinoma, and biliary pancreatitis did not correlate with clonorchiasis.

CONCLUSION: Infection rate of clonorchiasis was still high in patients with gastrointestinal diseases in Korea, and has not decreased very much during the last two decades. Cholangiocarcinoma was related to clonorchiasis, which suggested an etiological role for the parasite.

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Key words: *Clonorchis sinensis*; Epidemiology; Cholangiocarcinoma; Korea; Multicenter study; Clonorchiasis

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INTRODUCTION

Clonorchiasis is a parasitic infection caused by *C. sinensis,* and is one of the most prevalent endemic diseases in eastern Asia[1-3]. According to a report by World Health Organization (WHO) and International Agency for Research on Cancer in 1994, about 7 million people in the world were infected with *C. sinensis*[4]. In Korea, the stool egg-positive rate for *C. sinensis* has decreased dramatically from 4.6% in 1971 to 2.7% in 1986, after the introductioin of praziquantel, but the rate remained at 1.4% in 1997[5-8]. The high prevalence rate of clonorchiasis in Korea results from a long tradition of consuming raw freshwater fish and/or shellfish[9].

Infection rate in Korea differs from one major river basin to another. According to a national survey conducted in 1981, the stool egg-positive rate for *C. sinensis* in people of southern river basins (Nakdong, Yeongsan, and Seomjin Rivers) was 17%-40%, while that in people of middle river basins (Han, Geum, and Dangjim-Mankyong Rivers) was lower at 8%-12%[10]. However, there have been no data on infection rate among people in other river basins in middle and eastern areas (North Han, Bulyeong-Wangpi, and Namdae-Yeonggok-Osip Rivers).

Fecal examination for eggs has bee used in population-based studies for diagnosis of clonorchiasis[11]. However, this method has low sensitivity, which results in lower prevalence rates. Other methods for diagnosis include intradermal test using diluted antigens of *C. sinensis*[12], ELISA for circulating antibody against the parasite[13], radiological studies of the liver[14], and bile examination for eggs, metacercariae and cercariae. Of these, intradermal test is the easiest to perform, but has low specificity because of cross-reactivity with other parasites such as *Paragonimus westermani*[12]. Diffuse dilatation of the intrahepatic bile ducts detected by abdominal ultrasonography (US), computed tomography (CT), or cholangiography can easily establish clonorchiasis. In addition, detection of eggs in bile collected by endoscopic or percutaneous biliary drainage can lead to a definite diagnosis.

Adult worms of *C. sinensis* migrate from the common bile duct to peripheral intrahepatic bile duct, and remain there for 20-30 years causing chronic persistent infection[15]. In humans, clinical manifestations of light parasite loads are often asymptomatic. On the other hand, chronic infection with heavy parasite loads has been associated with various hepatobiliary diseases, such as biliary obstruction, recurrent pyogenic cholangitis[16], hepatolithiasis[17,18], and cholangiocarcinoma[19-21]. According to several experimental and clinical studies, clonorchiasis has been associated with carcinogenesis in the bile duct mucosa[4,19,22]. Adult worms, eggs, or mucoid material after infection can also be the nidus of hepatolithiasis[16,23,24]. Although there have been several studies on association between clonorchiasis and several hepatobiliary diseases, there has been no recent nationwide multicenter study in endemic areas and no investigation on prevalence and infection rates after raw freshwater fish and/or shellfish ingestion.

Therefore, we conducted a prospective nationwide multicenter study to investigate infection rate of *C. sinensis* in patients with gastrointestinal symptoms, and

the relation of *C. sinensis* infection with hepatobiliary diseases in 26 secondary and tertiary hospitals in Korea.

MATERIALS AND METHODS

Subjects

This prospective study was conducted in 26 secondary and tertiary hospitals in South Korea from March to April 2005. Subjects included consecutive patients with gastrointestinal symptoms who were admitted to the Department of Internal Medicine during the study period. Gastrointestinal symptoms were defined as the presence of any of the following: nausea, vomiting, diarrhea, constipation, abdominal pain, heartburn, dyspepsia, jaundice, indigestion, and fecal incontinence. Patients were excluded if they had been admitted with non-gastrointestinal symptoms, admitted more than twice during the study period, unable to give a thorough history, < 14 years old, and declined to participate in this study.

The institutional review board of each participating hospital approved this study. Informed consent for participation in this study was obtained from each patient included in the study.

Questionnaires

Upon admission, attending physicians filled out a structured questionnaire for each subject after a medical interview. The questionnaire included the following information: rivers nearest to the birthplace or place of current residence in order of decreasing duration, history of eating raw freshwater fish and/or shellfish, including the time, place and species of the fish and/or shellfish consumed, past history of clonorchiasis and treatment including type and duration, and past history of hepatobiliary diseases. Rivers nearest to the birthplace or place of current residence included 10 major rivers in South Korea: Nakdong, South Han, North Han, Geum, Yeongsan, Seomjin, Mangyong-Dongjin, Hyeongsan, Bulyeong-Wangpi, and Namdae-Yeongok-Osip.

The questionnaire also included close-ended questions such as: (1) did you (the patient him/herself) know that clonorchiasis can be acquired by ingesting raw freshwater fish? (2) Did you know that clonorchiasis can also be acquired by eating freshwater shellfish? (3) Did you know that clonorchiasis can be transmitted *via* kitchen knives and/or towels? (4) Did you know clonorchiasis can be transmitted by unwashed hands of raw freshwater fish handlers? (5) Did you know clonorchiasis can be prevented by eating fully cooked freshwater fish?

Diagnostic methods for C. sinensis infection

After admission, all the patients underwent laboratory tests, which included complete blood count with differential count, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase, gamma-glutamyl transpeptidase (GGT), and total bilirubin. When transabdominal US or abdominal CT was done after admission or within 6 mo of admission, presence of dilatation of the intrahepatic bile ducts and/or extrahepatic bile duct was recorded.

Diagnosis of clonorchiasis was based on the presence of one or more of the following findings: (1) detection of *C. sinensis* eggs, metacercariae, or adult worms in stools collected during admission; (2) presence of induration with area of 60 mm^2 or greater on the forearm after skin test with an intradermal injection of diluted crude antigen of *C. sinensis*; (3) positive for serum antibodies to *C. sinensis* using ELISA; (4) detection of *C. sinensis* eggs, metacercariae, or adult worms in bile collected during percutaneous transhepatic biliary drainage or endoscopic nasobiliary drainage; (5) finding of diffuse dilatation of intrahepatic bile ducts in transabdominal US, abdominal CT, or cholangiography; and (6) detection of *C. sinensis* infection in stools or bile examination, and/or presence of positive intradermal test described in medical records. To investigate the possible association between clonorchiasis and hepatobiliary diseases, medical records of each patient were reviewed for diagnosis of the following diseases: cholangitis, choledocholithiasis, gallbladder stones, hepatocellular carcinoma, cholangiocarcinoma, gallbladder cancer, biliary pancreatitis, and alcoholic pancreatitis.

Statistical analysis

Overall infection rate of *C. sinensis* and that according to the river basins were calculated. Infection rates between raw freshwater fish and/or shellfish eaters and non-eaters were also compared. Level of knowledge on transmission and prevention of clonorchiasis was assessed. By using the χ^2 test and independent *t* test, the differences between infected and non-infected patients were assessed with regard to the presence of peripheral eosinophilia and abnormal liver function tests. Sensitivities of various diagnostic methods used in this study were evaluated.

Association with hepatobiliary diseases was evaluated for infected and non-infected patients by using the χ^2 test. Association between clonorchiasis and cholangiocarcinoma among raw freshwater fish and/or shellfish eaters from high prevalence river basins (Nakdong, Yeongsan, Seomjin, and Hyeongsan Rivers) and lower prevalence river basins (the remainder) was assessed using the *t* test and odds ratio. After correction for regions, Cochran's Mantel-Haenszel χ^2 test was used to evaluate the association between clonorchiasis and cholangiocarcinoma. SPSS (version 12.0 for Windows; Chicago, IL, USA) was used for statistical analysis and $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Subjects included 3080 patients from 26 hospitals. Number of patients according to rivers nearest to the birthplace or place of current residence was 947 (31.5%) in Nakdong, 774 (25.7%) in South Han, 270 (9.0%) in North Han, 303 (10.1%) in Geum, 137 (4.6%)

Table 1 Distribution of patients according to rivers nearest to the birthplace or place of current residence *n* (%)

Rivers	Number of patients
Nakdong	947 (31.5)
South Han	774 (25.7)
North Han	270 (9.0)
Geum	303 (10.1)
Yeongsan	137 (4.6)
Seomjin	97 (3.2)
Mangyong-Dongjin	266 (8.8)
Hyeongsan	145 (4.8)
Bulyeong-Wangpi	6 (0.2)
Namdae-Yongok-Osip	64 (2.1)
Not answered	71
Total	3080

in Yeongsan, 97 (3.2%) in Seomjin, 266 (8.8%) in Mangyong-Dongjin, 154 (4.8%) in Hyeongsan, six (0.2%) in Bulyeong-Wangpi, 64 (2.1%) in Namdae-Yeongok-Osip Rivers and 71 unanswered (Table 1). There were 1953 male and 1127 female patients. Male to female ratio was 1.7:1. Mean age of the patients was 58.2 years old (range, 14-98).

Past history of raw freshwater fish and/or shellfish ingestion and C. sinensis infection

The number of patients with past history of raw freshwater fish and/or shellfish ingestion was 1140 (37.3%) out of 3055 of those who answered the questionnaire, while there were 191 (62.7%) patients with no past history of ingestion, and there were 25 unanswered questionnaires. Of those with a past history of ingestion, 156 out of 1140 patients (13.9%) ingested only once, 318 patients (28.3%) two to four times, and 648 patients (57.8%) more than five times. Initial time of raw freshwater fish and/or shellfish ingestion was within 10 years in 278 patients (25.5%), 11-20 years ago in 207 (19.0%), 21–30 years ago in 179 (16.4%), > 30 years ago in 427 (39.1%), and 49 questionnaires were unanswered (Table 2).

In 1140 patients with a past history of ingestion, river basins nearest to the place of ingestion were Nakdong for 394 patients (35.2%), South Han for 156 (13.9%), North Han for 207 (18.5%), Geum for 84 (7.5%), Yeongsan for 47 (4.2%), Seomjin for 65 (5.8%), Mangyong-Dongjin for 78 (7.0%), Hyeongsan for 54 (4.8%), Bulyeong-Wangpi for three (0.3%), Namdae-Yeongok-Osip Rivers for 32 (2.9%), and 20 questionnaires were unanswered (Table 3).

Only 150 (5.0%) patients had been diagnosed with clonorchiasis in the past. The number of patients without a past history of diagnosis or treatment was 2880 (95.0%) and 50 questionnaires were unanswered. Of those 150 patients with a past history of clonorchiasis, 120 (81.6%) had received eradication therapy, 14 (9.5%) did not receive any therapy, 13 (8.8%) had undergone treatment without definite diagnosis, and three did not answer. There were 657 patients (21.3%) with a past history of hepato-biliary diseases. Of these patients, 395 (60.4%) had bile duct stones, 118 (18.1%) cholangitis, 64 (9.6%) cholangiocarcinoma, 116 (17.6%) jaundice of uncertain cause, and 112 (16.5%) pancreatitis.

Table 2 Frequency and time of raw freshwater fish or snail ingestion among the patients with positive history *n* (%)

Characteristics	Number of patients
History of ingestion	
Present	1140 (37.3)
Frequency	
Once	156 (13.9)
2-4 times	318 (28.3)
⩾ 5 times	648 (57.8)
Unknown or not answered	18
Time of first ingestion	
0-10 yr ago	278 (25.5)
11-20 yr ago	107 (19.0)
21-30 yr ago	179 (16.4)
⩾ 30 yr ago	427 (39.1)
Unknown or not answered	49
None	1915 (62.7)
Not answered	25
Total	3080

Table 3 Distribution and infection rate of the patients with positive history of raw freshwater fish or snail ingestion according to river basins nearest to place of residence

River basins	Patients, *n* (%)	Infected patients (*n*)	Infection rate (%)
Nakdong	394 (35.2)	132	33.5
South Han	156 (13.9)	12	7.7
North Han	207 (18.5)	15	7.2
Geum	84 (7.5)	7	8.3
Yeongsan	47 (4.2)	19	40.4
Seomjin	65 (5.8)	14	21.5
Mangyong-Dongjin	78 (7.0)	10	1.8
Hyeongsan	54 (4.8)	24	44.4
Bulyeong-Wangpi	3 (0.3)	0	0
Namdae-Yongok-Osip	32 (2.9)	4	12.5
Not answered	20	3	
Total	1140	238	20.9

Questionnaire on route of C. sinensis infection

Of 3049 patients who answered the questionnaire, 2464 (80.8%) knew that clonorchiasis can be acquired by ingesting raw freshwater fish and 1629 (53.3%) knew that clonorchiasis can also be acquired by eating raw freshwater shellfish. Also, 1141 (47.2%) knew that clonorchiasis can be transmitted *via* kitchen knives and/or towels and 1192 (39.1%) acknowledged that clonorchiasis can be transmitted by unwashed hands of raw freshwater fish handlers. In addition, 2371 patients (77.8%) knew that clonorchiasis can be prevented by eating fully cooked freshwater fish (Table 4).

Presence of C. sinensis infection

Diagnosis of infection: Of 3080 patients admitted to the Department of Internal Medicine during the study period, 396 (12.9%) had been diagnosed with clonorchiasis. Stool examination was positive for *C. sinensis* eggs, metacercariae, or adult worms in 55 patients. Intradermal test was positive in 225 patients and serum antibodies to *C. sinensis* using an ELISA

Table 4 Answers to questionnaires regarding knowledge on route of *C. sinensis* infection

Questions	patients with "Yes" *n* (%)	Patients with "No" *n* (%)	Number of not answered (*n*)
Did you (the patient him/herself) know that clonorchiasis can be acquired by ingesting raw freshwater fish?	2464 (80.8)	585 (19.2)	31
Did you know that clonorchiasis can also be acquired by eating freshwater shellfish?	1626 (53.3)	1423 (46.7)	31
Did you know that clonorchiasis can be transmitted via kitchen knifes and/or towels?	1441 (47.3)	1608 (52.7)	31
Did you know clonorchiasis can be transmitted by unwashed hands of raw freshwater fish handlers?	1192 (39.1)	1855 (60.9)	33
Did you know that clonorchiasis can be prevented by eating fully cooked freshwater fish?	2371 (77.8)	676 (22.2)	33

Table 5 Sensitivities of various diagnostic modalities for detection of clonorchiasis

Diagnostic modalities	Infected persons, who were tested (*n*)	Positive results (*n*)	Sensitivity (%)
Fecal exam for eggs	321	55	17.1
ELISA for circulating antibody	362	157	43.40
Intradermal test	302	225	74.50
Examination of collected bile	134	14	10.40
Radiologic findings	295	34	11.50

Table 6 Distribution of patients with clonorchiasis according to age group *n* (%)

Age group (yr)	Number of patients (*n*)	Number of patients with clonorchiasis
10-19	26	0 (0)
20-29	107	7 (6.5)
30-39	222	25 (11.3)
40-49	463	79 (17.1)
50-59	695	99 (14.2)
60-69	831	113 (13.6)
70-79	575	61 (10.6)
⩾ 80	161	12 (7.5)
Total	3080	396

were positive in 157 patients. In 14 patients, *C. sinensis* eggs, metacercariae, or adult worms were detected in bile collected during percutaneous transhepatic biliary drainage or endoscopic nasobiliary drainage. Diffuse dilatation of intrahepatic bile ducts in transabdominal US, abdominal CT, or cholangiography was found in 34 patients. *C. sinensis* infection in stools or bile examination and/or presence of positive intradermal test was described in the medical records of 150 patients.

Sensitivities of the diagnostic tests were highest for intradermal test (74.5%) and second highest for serum antibodies to *C. sinensis* using an ELISA (43.4%) (Table 5).

Among patients with clonorchiasis, there was no patient younger than 19 year old. There were seven patients (6.5%) out of 107 in their twenties, 25 (11.3%) out of 222 in their thirties, 79 (17.1%) out 463 in their forties, 99 (14.2%) out of 695 in their fifties, 113 (13.6%) out of 831 in their sixties, and 61 (10.6%) out of 575 in their seventies. There were 12 (7.5%) out of 161 patients older than 80 years (Table 6).

Table 7 Presence of clonorchiasis according to history of raw freshwater fish ingestion *n* (%)

	Number of patients with clonorchiasis
Total number of patients (*n* = 3080)	396 (12.9)
Patients with positive history of raw freshwater fish ingestion (*n* = 1140)	238 (20.9)
Patients without raw freshwater fish ingestion (*n* = 1940)	158 (8.1)

Distribution of infected patients according to river basins from where ingested raw freshwater fishes originated: Of 1140 patients with a history of raw freshwater fish and/or shellfish ingestion, 238 (20.9%) had been diagnosed with clonorchiasis. Also, there was evidence of clonorchiasis in 157 out of 1940 patients (6.5%) who had no history of ingestion or had not answered the questionnaire (Table 7).

Of 1120 patients who answered, the river basin nearest to the place of raw freshwater fish or shellfish ingestion was Nakdong for 132 patients, South Han for 12 patients, North Han for 15 patients, Geum for seven patients, Yeongsan for 19 patients, Seomjin for 14 patients, Mangyong-Dongjin for 10 patients, Hyeongsan for 24 patients, Bulyeong-Wangpi for none, and Namdae-Yeongok-Osip Rivers for four patients (Table 4). The river basin with highest infection rate was Hyeongsan (44.4%). Other river basins in decreasing order of infection rate were Yeongsan (40.4%) and Nakdong (33.5%).

Laboratory findings: Eosinophilia in the peripheral blood (> 400/mm^3) was found in 65 of 389 patients with clonorchiasis (16.7%), while it was found in 250 of 2617 patients (9.6%) without clonorchiasis (P = 0.000) (Table 8). Serum alkaline phosphatase was 304.8 ± 418.35 U/L in 382 patients with clonorchiasis, but 234.4 ± 350.81 U/L in 2611 patients without clonorchiasis (P = 0.002). However, levels of AST, ALT, GGT, and total bilirubin were not significantly different between

Table 8 Comparison of laboratory findings between patients with clonorchiasis and without clonorchiasis

Laboratory findings	With clonorchiasis (n = 396)		Without clonorchiasis (n = 2684)		P
	Number of patients	mean ± SD	Number of patients	mean ± SD	
Eosinophilia[1]	65	NA	250	NA	0
AST (U/L)	393	104.5 ± 269.56	2671	110.7 ± 263.59	0.665
ALT (U/L)	393	113.5 ± 254.01	2669	107.7 ± 258.17	0.68
Alkaline phosphatase (U/L)	382	304.8 ± 418.35	2611	234.4 ± 350.81	0.002
γ-glutamyl transpeptidase (U/L)	362	200.0 ± 261.19	2233	187.0 ± 440.01	0.585
Total bilirubin (mg/dL)	392	3.07 ± 17.246	2668	3.28 ± 5.401	0.81

[1]> 400/mm^3 in peripheral blood; NA: Not available.

Table 9 Association between hepatobiliary diseases and presence of clonorchiasis *n* (%)

Hepatobiliary diseases	Patients with clonorchiasis (n = 396)	Patients without clonorchiasis (n = 2684)	P
Cholangitis	32 (8.0)	242 (9.0)	NS
Bile duct stones	92 (23.2)	716 (26.7)	NS
Gallstone	45 (11.4)	340 (12.7)	NS
Intrahepatic bile duct stones	13 (3.3)	107 (4.0)	NS
Extrahepatic bile duct stones	34 (8.6)	269 (10.0)	NS
Hepatitis	100 (25.3)	650 (24.2)	NS
Hepatitis B virus	58 (14.6)	336 (12.5)	NS
Hepatitis C virus	5 (1.3)	66 (2.5)	NS
Alcoholic	25 (6.3)	159 (5.9)	NS
Toxic	5 (1.3)	40 (1.5)	NS
Autoimmune	1 (0.3)	5 (0.2)	NS
Other causes	6 (1.5)	44 (1.6)	NS
Hepatocellular carcinoma	51 (12.9)	391 (14.6)	NS
Cholangiocarcinoma	34 (8.6)	145 (5.4)	0.015
Gallbladder cancer	9 (2.3)	75 (2.8)	NS
Biliary pancreatitis	6 (1.5)	71 (2.6)	NS

NS: Not significant.

the two groups (Table 8).

Association between clonorchiasis and hepatobiliary diseases: When prevalence of various hepatobiliary diseases was evaluated between patients with and without clonorchiasis, a statistically significant difference was found only for cholangiocarcinoma [34 (8.6%) *vs* 145 (5.4%), P = 0.015] (Figure 1A). There was no significant difference regarding cholangitis, bile duct stones, hepatitis, hepatocellular carcinoma, gallstone pancreatitis, and gallbladder cancer between patients with and without clonorchiasis (Table 9).

Patients were divided into two groups according to regions of the river basins nearest to the place of residence, and analyzed for association between presence of clonorchiasis and cholangiocarcinoma. Rivers of the southern region of the Korean Peninsula included Nakdong, Yeongsan, Seomjin and Hyeongsan Rivers and those of the middle region included South Han, North Han, Geum, Bulyeong-Wangpi, Namdae-Yeongok-Osip, and Mangyong-Dongjin Rivers (Figure 2). Clonorchiasis was present in 189 of 560 patients (33.8%) from the southern region, but in only 48 of 560 patients (8.6%) from the middle region (P = 0.000). While cholangiocarcinoma was found in 39 of 560 patients (7.0%) from the southern region, it was found in 19 of 560 patients (3.4%) from the middle region (P = 0.007) (Figure 1B).

In both southern and middle region groups, there was a significant association between presence of clonorchiasis and cholangiocarcinoma [P = 0.005, odds ratio: 4.136 (95% CI, 1.422-12.030] and P = 0.040, odds ratio: 1.961 (95% CI, 1.020-3.773), respectively]. Even after correction for regional influence, there was a significant association between presence of clonorchiasis and cholangiocarcinoma [P = 0.003, common odds ratio: 2.289 (95% CI, 1.297-4.038)] (Table 10, Figure 1B).

Twenty-four of 34 patients with clonorchiasis and cholangiocarcinoma had a history of raw freshwater fish and/or shellfish ingestion. Fifteen of these patients (62.5%) had ingested raw freshwater fish and/or shellfish 30 years ago, seven patients did so within last 10 years, and the other two patients ingested raw freshwater fish and/or shellfish between 21 and 30 years ago.

DISCUSSION

In this prospective study of Korean patients who had been admitted with gastrointestinal symptoms, 37.2% had a history of ingesting raw freshwater fish and/or shellfish more than once. Many Koreans still enjoy raw freshwater fish and/or shellfish and most do so more than once in his/her lifetime.

More than 80% of the patients knew that ingesting raw freshwater fish can result in clonorchiasis and 78% answered that eating fully cooked freshwater fish can prevent clonorchiasis. However, only 50% knew that eating raw freshwater shellfish could result in clonorchiasis. About 40%-50% of the patients also knew that clonorchiasis can be transmitted through kitchen knives, towels, kitchen boards, and/or unwashed hands of the cook or handler. Many people knew the transmission route of clonorchiasis, but still enjoyed eating raw freshwater fish and/or shellfish. Perhaps, these people believed that clonorchiasis can be easily treated with oral medication and clonorchiasis will not result in serious hepatobiliary diseases.

Since the objective of this study was to evaluate the association between clonorchiasis and various hepatobiliary diseases, rather than to evaluate epidemiology of clonorchiasis in the Korean population,

Table 10 Association between prevalence of clonorchiasis and cholangiocarcinoma according to regions of the rivers (n = 560)

	Rivers in southern region[1]			Rivers in middle region[2]		
	With cholangiocarcinoma	Without cholangiocarcinoma	χ^2 (P)	With cholangiocarcinoma	Without cholangiocarcinoma	χ^2 (P)
With clonorchiasis, n (%)	5 (10.4)	43 (89.6)	7.902 (0.005)	19 (10.1)	170 (89.9)	4.2 (0.04)
Without clonorchiasis, n (%)	14 (2.7)	498 (97.3)		20 (5.4)	351 (94.6)	
Odd ratio	4.136 (95% CI 1.422-12.030)			1.961 (95% CI 1.020-3.773)		
Common odds ratio[3]	2.289 (95% CI 1.297-4.038)					

[1]Nakdong, Yeongsan, Seomjin, Hyeongsan Rivers; [2]South Han, North Han, Geum, Bulyeong-Wangpi, Namdae-Yeongok-Osip, Mangyong-Dongjin Rivers; [3]P = 0.003, result of Cochran's Mantel-Haenzel test adjusted area.

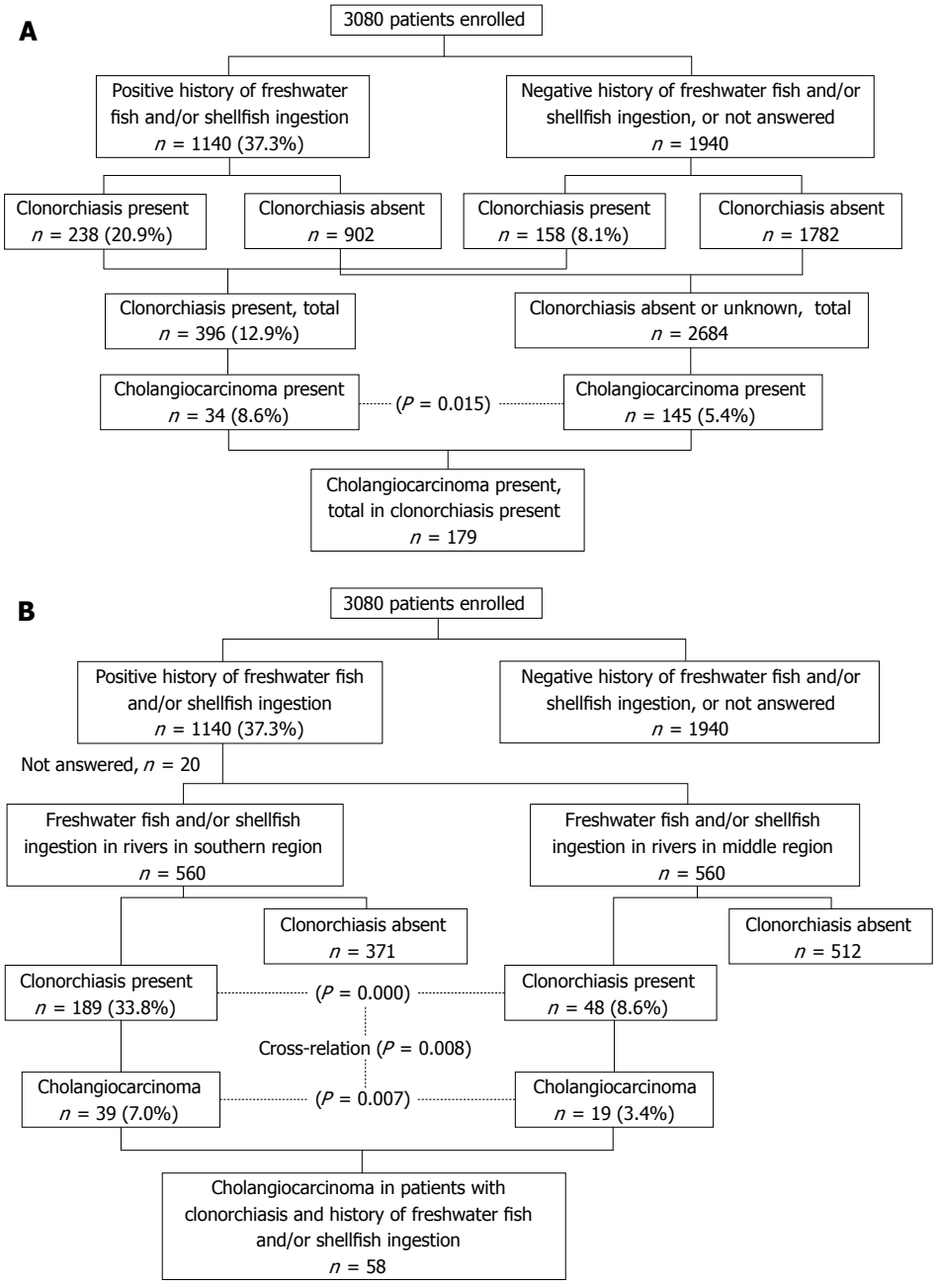

Figure 1 Flowchart for the comparison of the frequency of cholangiocarcinoma. A: According to the presence or absence of clonorchiasis; B: According to the place of residence.

diagnosis of clonorchiasis was based not only on positive laboratory findings, but also on presence of peripheral intrahepatic bile duct dilatation and clonorchiasis documented in medical records. Of 3080 patients admitted with gastrointestinal symptoms, 12.9% had been diagnosed with clonorchiasis. It is apparent that clonorchiasis is still prevalent in the Korean population. In the past, epidemiological studies of clonorchiasis in Korea have been based on stool examination and/or intradermal tests[11,12]. One epidemiological study in 1969

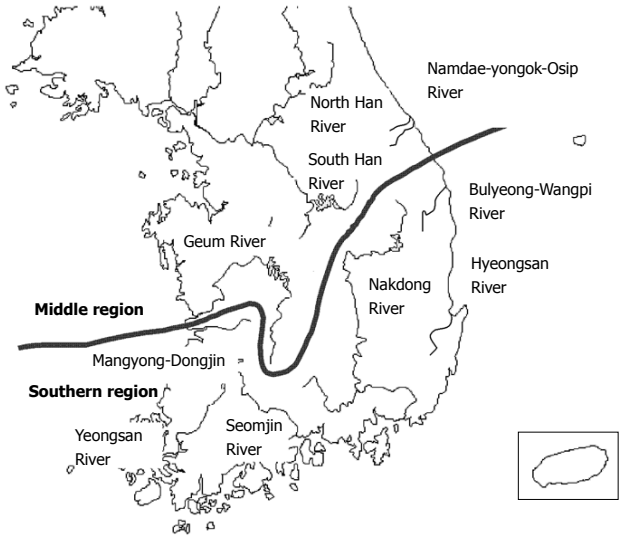

Figure 2 Rivers in South Korea. The basins of rives are divided into middle and southern regions.

utilizing cellophane thick smear revealed that 11.6% of 3880 subjects were infected with *C. sinensis*[25]. Another study in 1973 using an intradermal test showed an infection rate of 21.1%[5]. In 1981, stool examination of 13 000 Koreans from seven river basins demonstrated a clonorchiasis infection rate of 21.5%. From national surveys of clonorchiasis done every 5 years since 1971, the Ministry of Health and Welfare have reported an infection rate of 1.8%-4.6% in the Korean population[2]. Although the present study enrolled patients admitted with gastrointestinal symptoms, clonorchiasis seemed still prevalent with an infection rate of 12.9%. About one out of five patients who had ingested raw freshwater fish had clonorchiasis. Also, 6.5% of those without a history of raw freshwater fish ingestion had clonorchiasis, which implied that there might be routes of infection other than ingestion of raw freshwater fish or shellfish.

In a previous study, people from river basins in southern region of the Korean Peninsula showed higher infection rates when compared to those from river basins in the middle region (17.3%-40.2% *vs* 8.0%-17.3%, respectively)[10]. The results of the present study were similar to those of the previous study. While 33.8% of patients from river basins in southern region were infected with clonorchiasis, only 8.3% of those from the middle region were infected.

Among various methods used to diagnose clonorchiasis in the present study, intradermal tests showed the highest sensitivity of 74.5%, followed by detection of serum antibodies using ELISA, with a sensitivity of 46.4%. These two methods are limited by cross-reactivity and low specificity. Stool examination and bile cytology for adult worms and/or eggs have high specificity, but low sensitivity of 10%-12%. Radiological findings of intrahepatic bile duct dilatation also showed low sensitivity of 11.5%. In order to increase sensitivity, more than two diagnostic studies are needed.

Eosinophilia was found in 16.7% of patients with infection, while it was found in 9.6% of patients without clonorchiasis. Mean level of serum alkaline phosphatase was 304 U/L in patients with infection and 234.4 U/L in those without clonorchiasis. When laboratory tests during admission show eosinophilia with elevated alkaline phosphatase, clonorchiasis should be considered. There was no significant difference regarding other laboratory tests such as AST, ALT, GGT, and total bilirubin. Therefore, clonorchiasis cannot be excluded by liver function test only.

Adult worms of *C. sinensis* attach themselves with suckers to the walls of peripheral intrahepatic bile ducts. Long-term infection with *C. sinensis* is associated with various hepatobiliary diseases. It has been reported that cholangiocarcinoma has originated from papillary or adenomatous hyperplasia of the bile ducts infected with *C. sinensis*[26]. In a recent case-control study of Korean patients, peripheral intrahepatic bile dilatation or positive serum antibodies has been a risk factor for cholangiocarcinoma[27]. In the present study, there was no association of cholangitis and bile duct stones with clonorchiasis. Even after dividing bile duct stones into intrahepatic, extrahepatic, and gallbladder stones, there was no association between bile duct stones and clonorchiasis. In the present study, in patients admitted with gastrointestinal symptoms, cholangitis and bile ducts stones were present in 9% and 26.7% of patients without clonorchiasis. This may explain the absence of association of these diseases with clonorchiasis. Neither gallstone pancreatitis nor hepatitis of various causes was associated with clonorchiasis. Also, hepatocellular carcinoma and gallbladder cancer showed no association with clonorchiasis. Similar to results of other studies[26,27], cholangiocarcinoma was associated with the presence of clonorchiasis.

River basins of the southern region showed a higher infection rate of *C. sinensis* than those of the middle region (33.8% *vs* 8.6%, $P = 0.000$). Also, river basins of the southern region showed a higher prevalence rate of cholangiocarcinoma compared to those of the middle region (7.0% *vs* 3.4%, $P = 0.007$). The odds ratio of patients with clonorchiasis for cholangiocarcinoma was 4.136 (95% CI, 1.422-12.030) in the southern region and 1.961 (95% CI, 1.020-3.773) in the middle region. Even after correction for regional influence, the odds ratio was 2.289. According to these data, there was a strong correlation between clonorchiasis and cholangiocarcinoma. These findings were similar to the results of a previous study on the Korean population[28].

Initial ingestion of raw freshwater fish or shellfish dated back to 20 years ago in 70.8% of 24 patients with clonorchiasis and cholangiocarcinoma. Long-term infestation with *C. sinensis* is associated with development of cholangiocarcinoma, therefore, clonorchiasis should be treated as soon as possible when suspected. In this prospective multicenter nationwide study, prevalence of clonorchiasis and the association between clonorchiasis and hepatobiliary diseases in the Korean population were evaluated. Unlike other intestinal nematode infections, clonorchiasis is still prevalent. This seems to result from the habit of raw freshwater fish and/or

shellfish ingestion. Since prevalence of clonorchiasis in river basins of the southern region was higher than other parts, there is an urgent need for public education to prevent further raw freshwater fish or shellfish ingestion. Also, the presence of clonorchiasis was associated with cholangiocarcinoma and is a risk factor for cholangiocarcinoma.

COMMENTS

Background

Clonorchiasis is an infection caused by the parasite *C. sinensis*, and has been one of the most important endemic diseases in eastern Asia. Clonorchiasis can cause a variety of gastrointestinal diseases such as bile duct obstruction, acute cholangitis, hepatolithiasis, and cholangiocarcinoma.

Research frontiers

Important areas in the research field related to this article are development of more rapid and convenient diagnostic modalities and antihelminthic vaccines, and investigation of mechanisms by which *C. sinensis* causes cellular injury.

Innovations and breakthroughs

Despite public efforts and education, clonorchiasis is still present in the Korean population, and more so in certain regions of the country. Also, clonorchiasis is associated with cholangiocarcinoma and is a risk factor for cholangiocarcinoma.

Applications

To prevent clonorchiasis and cholangiocarcinoma associated with this parasite infection, there is a need for public education to prevent further raw freshwater fish or shellfish ingestion.

Terminology

Acute cholangitis is inflammation of bile duct that can cause fever, abdominal pain and abnormal blood test results. Hepatolithiasis refers to stone formation inside the liver. Cholangiocarcinoma is a certain type of liver cancer that originates from bile duct.

Peer review

This study showed an impressive epidemiology and way of infection of clornochiasis in riverside populations of Korea. The authors collected data from 26 hospitals in only 1 mo. This is an important contribution to the etiology of cholangiocarcionoma in this part of the world. Also, the results of this study can serve as a basis for public health initiatives for prevention and mass treatment of clonorchiasis.

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ORIGINAL ARTICLES

Treatment of chronic proliferative cholangitis with c-myc shRNA

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Abstract

AIM: To investigate the feasibility and effectiveness of c-myc shRNA in inhibiting the hyperplastic behavior and lithogenic potentiality of chronic proliferative cholangitis (CPC), in order to prevent stone recurrence and biliary restenosis.

METHODS: An animal model of CPC was established by giving intralumenally 0.5 mL of c-myc shRNA. Then, the effects of c-myc shRNA on hyperplastic behavior and lithogenic potentiality of CPC were evaluated by histological observation, immunohistochemistry, real-time PCR and Western blotting for c-myc, proliferating cell nuclear antigen (PCNA), procollagen Ⅲ, mucin 5AC, enzymatic histochemistry for β-glucuronidase, and biochemistry for hydroxyproline in the diseased bile duct.

RESULTS: Treatment with c-myc shRNA efficiently suppressed the hyperplasia of biliary epithelium, submucosal gland, and collagen fiber by inhibiting mRNA and protein expression of c-myc. More importantly, it decreased the lithogenic potentiality of CPC by inhibiting the expression of mucin 5AC and the secretion of endogenous β-glucuronidase. Further investigation indicated that c-myc shRNA-3 had a better inhibitory effect on CPC.

CONCLUSION: Treatment with c-myc shRNA-3 can control CPC and reduce the lithogenic potentiality of CPC.

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Key words: Chronic proliferative cholangitis; Hepatolithiasis; Recurrence; C-myc; Prevention

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Li FY, Cheng NS, Cheng JQ, Mao H, Jiang LS, Li N, He S. Treatment of chronic proliferative cholangitis with c-myc shRNA. *World J Gastroenterol* 2009; 15(1): 95-101 Available from: URL: http://www.wjgnet.com/1007-9327/15/95.asp DOI: http://dx.doi.org/10.3748/wjg.15.95

INTRODUCTION

Hepatolithiasis, a commonly encountered disease in the Asian-Pacific region, is more refractory to surgical treatment than most other benign diseases of the biliary tract[1,2]. Its therapeutic challenges include the difficulty in completely correcting the biliary stenosis and a high rate of stone recurrence, leading to a re-operative rate of 37.1%-74.4%[1-3]. Unfortunately, how to prevent the stone recurrence and biliary restenosis is still a problem to be solved in hepatobiliary surgery. Since hepatectomy cannot eliminate the possibility of stone recurrence, 16% of postoperative patients may develop new stones at other sites[3-5]. Therefore, surgery itself cannot achieve its long-term therapeutic effectiveness on hepatolithiasis[2-7]. In recent years, with a deeper understanding of the pathological changes in hepatolithiasis, the high stone recurrence rate and biliary restenosis rate in hepatolithiasis patents have been found to be related to the residual chronic proliferative cholangitis (CPC) after operation[8-11].

In the past, we paid too much attention to the improvement of surgical skills for treatment of hepatolithiasis, but failed to sufficiently recognize the connection of CPC to the formation of intraheptic calculi, and to pay enough attention to the treatment of residual CPC after removal of stones[3,12-14]. Thus, even though the stone is removed completely and the biliary tract stenosis is corrected, the residual CPC induced by the stone would still exist persistently and

extensively, which would facilitate formation of new stones by producing mucoprotein or by changing the lithogenic pathology and biliary stricture, which leads to cholestasis. Therefore, treatment of CPC after operation might increase its curative effect on hepatolithiasis. Unfortunately, there is no definitely effective therapy for CPC at present[15-17]. Since hepatolithiasis is a chronic proliferative disease, we designed this study to investigate the preliminary effectiveness of c-myc shRNA on hyperplastic behavior and lithogenic potentiality of CPC, expecting to prevent stone recurrence or biliary restenosis by controlling or eradicating CPC[18,19].

MATERIALS AND METHODS

Study design and surgical procedure

A total of 56 Sprague-Dawley rats weighing 220-250 g were randomly divided into six groups. (1) CPC group (n = 10) in which a 5-0 nylon thread was inserted into the common bile duct through the duodenal papilla[20]. (2) Four c-myc shRNA treatment groups (n = 10), in which a nylon thread was used as a guidwire and a 20 G veinous retaining needle was introduced into the common bile duct. Then, a total of 3×10^9 plaque-forming units (pfu) of four kinds of c-myc shRNA (shRNA-1, shRNA-2, shRNA-3, and a negative control sequence provided by Genesil Biotechnology Co. Ltd, Wuhan, China) in a total volume of 0.5 mL mediated by liposome 2000 (Invitrogen, USA) were respectively infused. (3) Sham operation (SO) group (n = 6) in which the common bile duct was dissected only. Transfection efficiency was detected after 48 h. One week later, all the rats were sacrificed with their common bile ducts removed, and fixed in liquid nitrogen and 10% formaldehyde for further tests.

Immunohistochemistry or immunofluorescence staining of c-myc and mucin 5AC

The avidin-biotin-peroxidase complex method was used to detect the expression of c-myc. Briefly, tissue sections were incubated overnight at 4℃ with primary antibody (Zymed Co, USA), followed by incubation with biotinylated second antibody for 1 h at 37℃. Expression of mucin 5AC was detected with immunofluorescence staining. Briefly, cryostat slides were incubated with primary antibodies (Santa Cruz Biotechnology, USA) at 4℃ overnight. After incubation with secondary antibody labeled with fluorescein at 37℃ for 1 h, the expression of mucin 5AC was observed under a fluorescent microscope at once.

Detection of c-myc and mucin 5AC by real time-PCR

Total RNA was extracted from the bile duct wall using Trizol (Gibco, USA). Reverse transcription was performed according to the manufacturer's instructions (Gibco, USA). Real-time analysis was performed on the cycle (Bio-Rad, Germany) using SYBR Green (TaKaRa, Dalian, China). Levels of target gene expression in the tested samples were normalized to the corresponding GAPDH mRNA transcript.

Detection of c-myc, PCNA and procollagen Ⅲ by Western blotting

After protein concentration was determined, 100 μg protein was loaded in each lane and subjected to 8% SDS-PAGE gel electrophoresis, then transferred to nitrocellulose membrane for immunoblotting. The blots were probed with antibodies against c-myc, proliferating cell nuclear antigen (PCNA) and procollagen Ⅲ (dilution 1:1000, Zymed Co, USA) overnight at 4℃. After washed with TBST, the membrane was incubated for 2 h with HRP-conjugated rabbit anti-rat secondary antibody (dilution 1:3000). Immunoreactive bands were visualized with enhanced chemiluminescence and captured on a X-ray film.

Enzymatic histochemical staining of endogenous β-glucuronidase in bile duct wall

The method of Ballantyne was used to perform enzymatic histochemical staining using nephthol-AS-SI-β-D-glucuronide (β-G; Sigma) as the substrate[21]. Briefly, cryostat sections were incubated at 37℃ for 1 h in a pH-4.95 solution containing the β-G substrate and hepatocyte nuclei were counterstained with methyl green for 3 min. Positive expression of endogenous β-G was observed as a rose-red signal in cytoplasm.

Assessment of hydroxyproline content (mg/g of bile duct)

Connective tissue in the blie duct was estimated by quantifying hydroxyproline, an amino acid found primarily in collagen, the principal component of extracellular matrix. The hydroxyproline content was detected as previously described[22].

Statistical analysis

All the data were presented as mean ± SD and analyzed using the SPSS10.0 software. Statistical analysis was conducted using the non-parametric ANOVA to evaluate the variance among more than two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Histopathological examination

Biliary epithelium mucosa in the CPC group was histologically characterized by papillary hyperplasia projection, which led to obstruction of the bile duct lumen. Analogical histological changes were observed in the negative control group. In contrast, proliferative degrees of biliary epithelium, submucosal gland, and collagen fiber in the three c-myc shRNA treatment groups were obviously lower than those in the CPC group, especially in the c-myc shRNA-3 group. More specifically, the degree of fibrous thickening in the diseased bile duct wall was obviously relieved after treatment with c-myc shRNA (Figure 1A-C).

Determination of c-myc and PCNA by immunohistochemistry, RT-PCR and Western blotting

To determine the anti-proliferative effect of c-myc

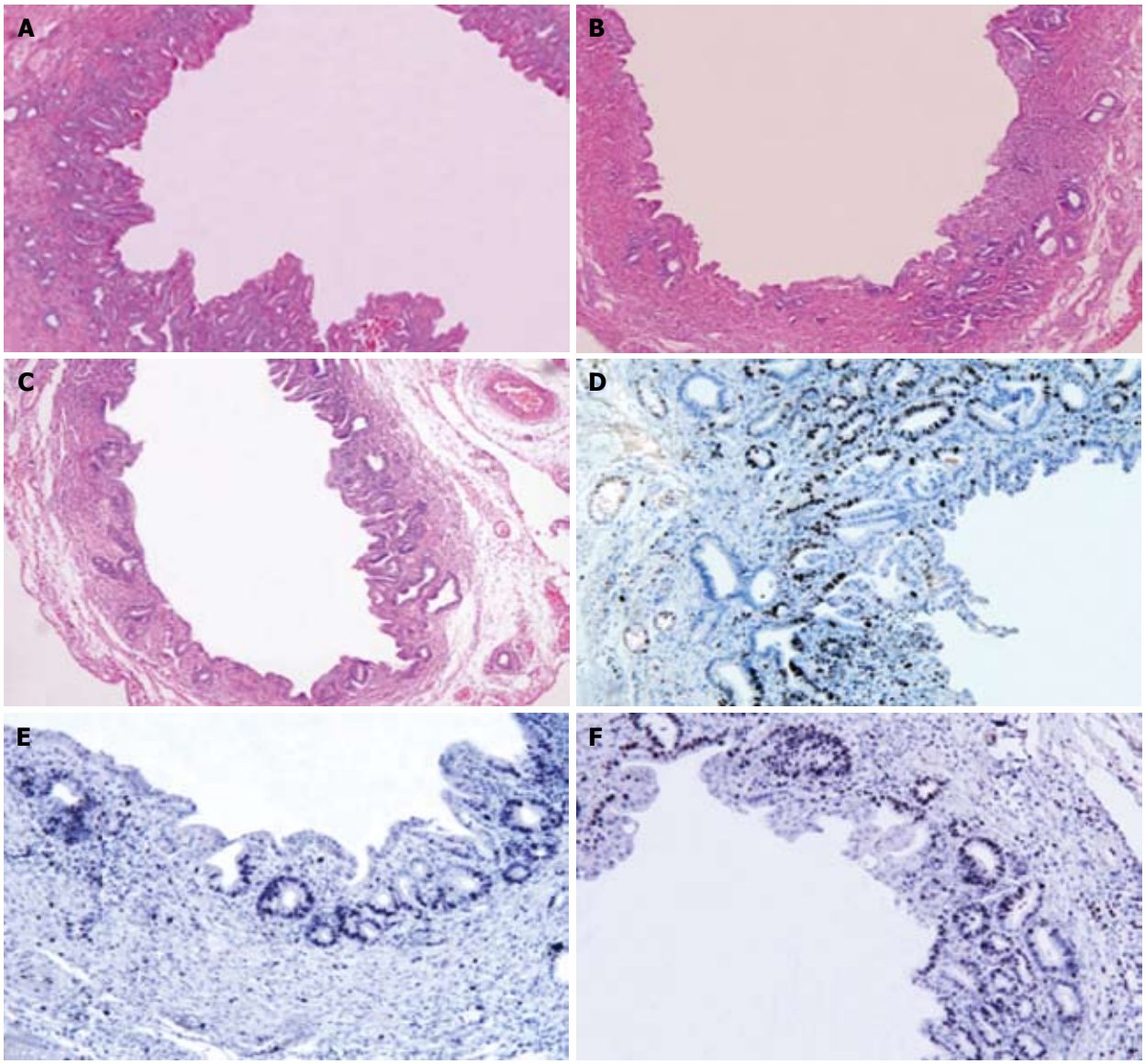

Figure 1 HE staining and immunohistochemistry for c-myc in CPC group (A, D), c-myc shRNA-3 treatment group (B, E), and c-myc shRNA-2 treatment group (C, F). Briefly, treatment with c-myc shRNA, especially with c-myc shRNA-3, can efficaciously inhibit hyperplasia of biliary epithelium, submucosal gland, collagen fiber, and down-regulate c-myc expression (A-C, × 50; D-F, × 100).

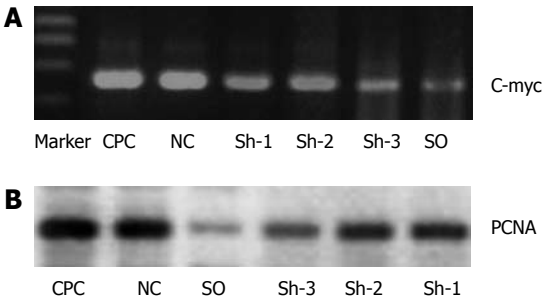

Figure 2 Real-time PCR (A) and Western blot (B) analysis of c-myc and PCNA expression in biliary duct wall.

shRNA, we compared the expression of c-myc, PCNA mRNA and protein in the diseased bile duct wall, revealing a remarkable decrease of c-myc, PCNA mRNA and protein expression in the c-myc shRNA treatment groups ($P < 0.0001$ and $P = 0.001$, respectively), c-myc shRNA-3 treatment group, but still significantly higher than that in the SO group (0.97 ± 0.28 *vs* 0.22 ± 0.09, $P < 0.0001$; 0.82 ± 0.22 *vs* 0.45 ± 0.08, $P = 0.011$). The mRNA and protein levels of c-myc, and PCNA did not differ significantly between the c-myc shRNA-1 and the c-myc shRNA-2 treatment groups. Also, the difference between the CPC group and the negative control group was not statistically significant (Table 1, Figures 1D-F and 2).

Detection of mucin 5AC expression by RT-PCR and immunohistochemistry

To probe the influence of c-myc shRNA on lithogenic potentiality of CPC, RT-PCR and Immunohistochemistry analysis of mucin 5AC were performed, showing a significant decrease of mucin 5AC mRNA and protein expression in the c-myc shRNA treatment groups, which was even more prominent in the c-myc shRNA-3 treatment group (0.42 ± 0.16), when compared with the

Table 1 HYP content and expression level of c-myc, PCNA, mucin5AC, procollagen Ⅲ

	CPC	shRNA-1	shRNA-2	shRNA-3	NC	SO
c-myc/GAPDH	6.21 ± 1.97	2.37 ± 0.77	2.93 ± 0.84	0.97 ± 0.28	6.57 ± 2.11	0.22 ± 0.09
P	< 0.0001	0.001	< 0.0001		< 0.0001	< 0.0001
PCNA/β-actin	3.20 ± 0.81	1.52 ± 0.28	1.83 ± 0.42	0.82 ± 0.22	2.71 ± 0.63	0.45 ± 0.08
P	< 0.0001	0.013	0.005		0	0.011
Mucin5AC/GAPDH	1.87 ± 0.47	0.96 ± 0.28	1.05 ± 0.30	0.42 ± 0.16	1.69 ± 0.41	0.12 ± 0.04
P	< 0.0001	0.004	0.002		< 0.0001	< 0.0001
Procol-Ⅲ/β-actin	4.79 ± 1.27	2.83 ± 0.85	2.39 ± 0.58	1.23 ± 0.35	5.40 ± 1.76	0.52 ± 0.13
P	0	0.001	0.006		< 0.0001	0.001
HYP content	1.29 ± 0.32	0.78 ± 0.16	0.83 ± 0.18	0.55 ± 0.13	1.41 ± 0.36	0.39 ± 0.08
P	0.003	0.041	0.028		0.001	0.038

P value was compared with c-myc shRNA-3 treatment group.

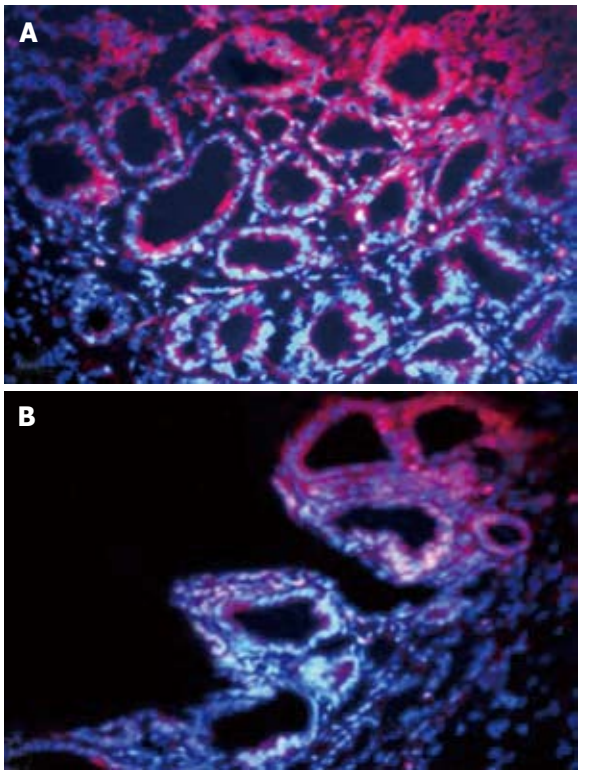

Figure 3 Immunofluorescence (A, B) and RT-PCR (C) analysis of mucin 5AC in bile duct wall. CPC group (A), c-myc shRNA-3 treatment group (B). Briefly, treatment with c-myc shRNA-3 can result in a more prominent down-regulation of mucin 5AC expression (A and B, × 400).

c-myc shRNA-1 and c-myc shRNA-2 treatment groups (0.96 ± 0.28, 1.05 ± 0.30; $P = 0.004$ and $P = 0.002$, Table 1, Figure 3).

Enzymatic histochemical staining of endogenous β-G

To further explore the influence of c-myc shRNA on pigment stone formation, we performed enzymatic histochemical staining of endogenous β-G, which showed a significant increase of endogenous β-G expression in the CPC group. However, β-G expression was significantly decreased in the c-myc shRNA-1, shRNA-2, and shRNA-3 treatment groups, but the difference was not significant in the three groups (Figure 4).

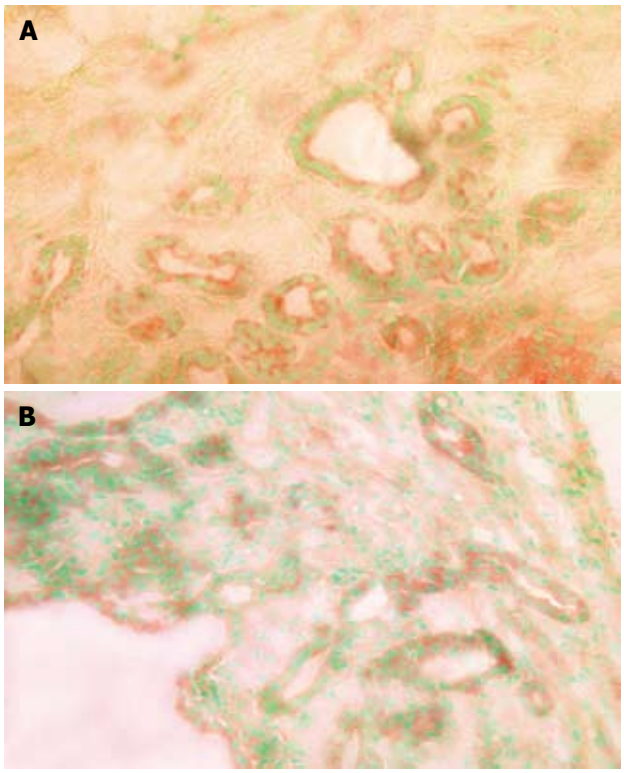

Figure 4 Enzymatic histochemistry staining of endogenous β-G (cryostat section, × 400) in CPC group (A) and c-myc shRNA-3 treatment group (B). A notable reduction of endogenous β-G was observed in the bile duct wall following c-myc shRNA treatment.

Western blot analysis of procollagen Ⅲ in biliary duct wall

To explore the influence of c-myc shRNA on collagen fiber proliferation, we examined the procollagen Ⅲ protein expression in the diseased bile duct. The bile duct in the CPC group displayed a very high level of procollagen Ⅲ protein expression. However, the expression of procollagen Ⅲ protein was significantly decreased after treatment with c-myc shRNA, which was even more prominent in the c-myc shRNA-3 treatment group (1.23

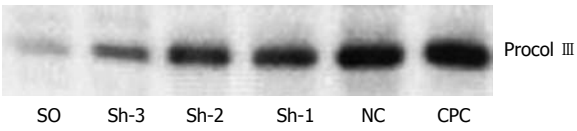

Figure 5 Western blot analysis of procollagen Ⅲ expression in biliary duct wall.

± 0.35 *vs* 2.83 ± 0.85 for the shRNA-1 treatment group, 2.39 ± 0.58 for the shRNA-2 treatment group, $P = 0.001$ and $P = 0.006$), although it was signifcantly higher than that in the SO group (Table 1, Figure 5).

Assessment of hydroxyproline (HYP) content (mg/g of bile duct)

Subsequently, quantitative determination of connective tissue in the diseased bile duct was performed, which showed that the HYP content in the CPC and negative control groups was approximately increased up to three-fold, when compared with the SO group (1.29 ± 0.32 and 1.41 ± 0.36 *vs* 0.39 ± 0.08). However, after treatment with c-myc shRNA, the HYP content was significantly decreased, especially in the c-myc shRNA-3 treatment group (0.55 ± 0.13 *vs* 0.78 ± 0.16 for the shRNA-1 treatment group, 0.83 ± 0.18 for the shRNA-2 treatment group, $P = 0.041$ and 0.028, respectively; Table 1).

DISCUSSION

Since hepatolithiasis is a common disease in Asia and its etiology remains obscure, it is quite difficult to treat and prevent this disease from its lithogenesis. According to its pathology, 75%-100% of hepatolithiasis patients in Asia are characterized by CPC. Increased attention has been paid in recent years to the cause-and-effect relationship of CPC and formation of stones[10,15-17]. Firstly, the stone can not only bring an inflammatory full-thickness penetrating damage to the local biliary duct mucosa in the obstructed part, but also lead to a down-stream mucosal injury in the distant bile duct as the stone and inflamed bile move downwards. Therefore, damage to the related biliary ducts caused by the stone is extensive, and not merely localized to the stone resident part. This is why stone-induced CPC exists widely after removal of the stone[11-16]. On the other hand, recurrent attacks of CPC would in turn facilitate formation of new stones by causing such lithogenic pathology changes as biliary stricture and biliary infection. Furthermore, recurrent CPC can be directly involved in formation of stones by producing mucoglycoproteins secreted by the proliferated submucosal gland. As we know, mucoglycoprotein is not only a contributing factor for intrahepatic stones but also a protein that can directly participate in formation of stone nucleation or reticular lithogenesis framework[23-26]. As mentioned above, a vicious cycle of CPC, biliary stricture and stone may develop. It is thereby reasonable that treatment of heaptolithiasis should be directed not only at removal of the stone and correction of the biliary stricture, but also at control of postoperative CPC, a key factor for this vicious cycle[12-16,19,20].

Recently, proto-oncogene c-myc has become an attractive target for anti-proliferative treatment of hypernomic proliferation diseases, because it is at the center of a transcription factor network that regulates cellular proliferation, replication, growth, differentiation, and apoptosis[27-30]. Specific blockage of the c-myc gene expression can partially inhibit cellular proliferation, thus efficiently preventing vascular restenosis after angioplasty by inhibiting endangium cell proliferation[27,28]. In this study, we also investigated the anti-proliferative effectiveness of c-myc shRNA on hypernomic proliferation behavior of CPC[17,18,29]. As expected, HE staining, immunohistochemistry, RT-PCR and Western blotting showed that treatment with c-myc shRNA could efficiently inhibit hyperplasia of the biliary epithelium, submucosal gland and collagen fiber by specifically blocking mRNA and protein expression of the proliferation-related gene c-myc and PCNA. The levels of c-myc, and PCNA mRNA and protein expression were much lower in the c-myc shRNA-3 treatment group than in the c-myc shRNA-1 and shRNA-2 treatment groups, which indicates that c-myc shRNA-3 may have a better anti-proliferative effect on CPC[9,15,27-30].

To analyze the effect of this gene therapy on the lithogenic potentiality of CPC, we compared the expression of mucin5AC and endogenous β-G in the diseased bile duct. Among the nine mucoglycoproteins identified so far, up-regulation of mucin5AC expression is considered to be closely related to the formation of stones[25,31], which is consistent with our findings. In the present study, the expression of mucin5AC mRNA and protein was significantly increased in the CPC group. However, the expression of mucin5AC was obviously decreased after treatment with c-myc shRNA, especially after treatment with c-myc shRNA-3, which suggests that c-myc shRNA can effectively inhibit the inactivation of such mucin genes as mucin5AC and secretion of muglycoprotein. It is noteworthy that reduced muglycoprotein helps decrease bile viscidity and aggregation or sedimentation of lithogenic ingredients in the bile, which might be significant in preventing stone recurrence[24,26,32]. The expression of endogenous β-G in the diseased bile duct was also obviously decreased after treatment with c-myc shRNA, which may be explained by the inhibitory effect of c-myc shRNA on the proliferation of biliary epithelium and submucosal gland. The inhibitory effect of c-myc shRNA on endogenous β-G would, to some degree, be helpful in preventing postoperative biliary stone recurrence[21,33].

Considering the potential anti-proliferative effect of c-myc shRNA on collagen fiber proliferation, c-myc shRNA treatment may prevent biliary tract restenosis secondary to CPC[8,9]. In our study, HE staining showed that collagen fiber proliferation was significantly lower in the diseased bile duct after treatment with c-myc shRNA than CPC, which suggests that the incidence of biliary tract stricture secondary to CPC can be reduced. Further comparison displayed that treatment with c-myc shRNA-3 demonstrated a better inhibitory effect on procollagen Ⅲ protein and HYP content than treatment

with shRNA-2 and shRNA-1, which indicates that c-myc shRNA-3 has a bright future in preventing bile duct fibrosis and biliary stricture[20,28,29,34].

In conclusion, anti-proliferative treatment with c-myc shRNA is likely to open a new feasible approach to the treatment of postoperative residual CPC. Furthermore, the inhibitory effects of c-myc shRNA on the lithogenic potentiality of CPC can assist in reducing postoperative recurrence of intrahepatic calculi. More importantly, this novel treatment would lay an experimental foundation of development of drugs for preventing stone recurrence after choledochoscopic lithotomy, at least in part, and reducing the incidence of reoperation and choledochoscopic lithotomy[2,13-18,20,30]. However, further study is needed on its long-term effect, related complications, and more efficient gene expression vectors before its clinical application[13,33,35].

COMMENTS

Background

In recent years, with a deeper understanding of the pathological changes in hepatolithiasis, the high stone recurrence rate has gradually been recognized, and is currently considered due to the postoperative chronic proliferative cholangitis (CPC). In this study, we investigated the inhibitory effect of c-myc shRNA on hyperplastic behavior and lithogenic potentiality of CPC.

Research frontiers

Multiple factors for lithogenesis of intrahepatic stones have brought enormous difficulties to its prevention and treatment and 75%-100% of hepatolithiasis patients in the Asian-Pacific regions are pathologically characterized by CPC, a key factor for preventing calculus recurrence. Treatment of CPC after operation might assist in increasing the curative effect on hepatolithiasis.

Innovations and breakthroughs

The high recurrence rate of intrahepatic stones is still a problem to be solved in hepatobiliary surgery. Since there is no effective medication for preventing stone recurrence after choledochoscopic lithotomy and for testing its pathology, stone recurrence and reoperation cannot be avoided. Our preliminary results showed that c-myc shRNA can inhibit hyperplastic behavior and lithogenic potentiality of CPC, thus laying an experimental foundation of development of drugs for preventing stone recurrence.

Applications

Intralumenal administration of c-myc shRNA is a promising therapeutic approach to CPC, and might assist in reducing the lithogenic potentiality of CPC.

Peer review

The authors of this paper investigated the efficacy of c-myc shRNA in ameliorating histological and molecular manifestations in an animal model of hepatolithiasis. The study was well designed and its findings are interesting and informative.

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BRIEF ARTICLES

Seroepidemiology of hepatitis A virus in Kuwait

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Abstract

AIM: To find the current seroepidemiology of hepatitis A virus (HAV) in Kuwait.

METHODS: A total of 2851 Kuwaitis applying for new jobs were screened.

RESULTS: HAV-positive cases were 28.8%; 59% were males and 41% were females. The highest prevalence was in the Ahmadi area. High prevalence was among the group of non-educated rather than educated parents. This is the first study in Kuwait demonstrating the shifting epidemiology of HAV.

CONCLUSION: This study reflects the need of the Kuwaiti population for an HAV vaccine.

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Key words: Hepatitis A virus; Fulminant Liver Failure; Hepatitis A virus vaccine; Kuwait

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INTRODUCTION

Hepatitis A virus (HAV) infection is often a self-limiting disease that can be associated with fulminant hepatic failure (FHF). The mortality rate tends to increase with age, in particular, when greater than 40 years of age[1]. Various data from the world shows that with the improvement in hygiene and quality of life there is a shift in the epidemiology of HAV to older people, exposing them to the risk of serious HAV infection.

Hepatitis A is often considered a benign disease in our area. The idea is that almost 100% of the adult population has been infected in early childhood[2]. However, there are no available data about the current epidemiology of the disease in Kuwait. The aim of this study was to find out the current sero-prevalence of this disease and to decide the need of the Kuwaiti population for HAV vaccine.

MATERIALS AND METHODS

Study population and serum collection

The study population was healthy adults attending a medical checkup that was required before applying for a new job. The study was performed in two places in Kuwait; the first one was the General Medical Council, which accepts adults from both sexes and all nationalities applying for civilian jobs. Only Kuwaiti nationals were included in the study. The second place was the Armed Forces Hospital, which accepts Kuwaiti adult males recruited for military service. The study population belong, to the total six governorates of the country of Kuwait, which includes the Capital, Hawali, Farwania, Mubarak, Ahmadi and Jahra.

The study was approved by the Ministry of Health and the local ethical committee of the Kuwait Institution for Medical Specialization (KIMS). Informed consent was obtained from each case. Each individual completed a questionnaire. Thereafter, 5 mL of blood was obtained. The identities of the subjects were kept confidential by assigning a code number for the questionnaire and the blood samples. The study was conducted during May 2003 to May 2004.

Laboratory data

Blood samples were collected from different centers and sent to the Virology Unit-Public Health Laboratories. An Axsym HAVAB 2.0 kit (Abbott Laboratories) was used for the detection of IgG anti-HAV from all samples. The procedure was followed as indicated by the manufacturer. In addition, the samples were tested for anti-hepatitis B surface antigen (HBsAg), anti-hepatitis C virus and anti-HIV.

Statistical analysis

Data are expressed using descriptive statistical methods, namely counts and percentages of screened subjects.

RESULTS

There were a total of 2851 Kuwaiti cases screened, 2216 were from the Medical Council and the remaining were from the Military Hospital.

Residence area

The screened cases were from the six governorates of Kuwait which included Farwania 528 cases (18.52%), Ahmadi 505 cases (17.71%), Hawali 467 (16.38%), Capital 433 (15.19%), Jahra 411 (14.42%), Mubarak 301 cases (10.56%) and 7.23% were from unidentified areas. Of 2851 cases screened, 816 (28.6%) cases were positive for HAV (Figure 1). The prevalence percentages of HAV in each governorate were higher in Ahmadi, 202 cases (24.5%); Farwania, 170 cases (20.6%); and Jahra, 164 cases (20%), than in others: Capital 89 cases (11%); Hawali, 84 cases (10%); Mubarak, 76 cases (9%); and 31 cases (3.8%) were unidentified. The 28% of cases which tested positive for HAV were in 94% of cases from the Medical Council and in 5% of cases from the Military Hospital.

Age and sex distribution

There were 481 (59%) males and 335 (41%) females. The prevalence of HAV cases in each age group was 24% (561) in the age group 18-27 years, 51% (213 cases) in the age group 28-40 years, and 56.5% (26 cases) in the age group 41-60 years (Table 1).

Education level

Of 2851 subjects screened, 1525 had both parents who were educated, while 412 had non-educated parents. The prevalence of HAV among the group with non-educated parents or a single educated parent was higher than the educated group (42%, 37% and 21%, respectively) (Figure 2). Of the 816 cases who had HAV, single parent education was determined as maternal education in 10%, while paternal education was 90%. Maternal education plays a greater role than paternal in the care of children and the family which is reflected in the social and health standards (Figure 3).

Risk factors

There was no risk factor in 2706 cases, household contacts with hepatitis in one case, surgery in two, blood transfusion in four, and unknown answer in 138 cases.

Association with other viral hepatitis

In this study, 2035 (71%) cases were not immune to HAV, 94% of them had no evidence of HBV or HCV infection. In 4.8% of cases, there was positive HBsAg serology, while 1.5% had positive anti-HCV results. Of 816 (28.6%) having immunity to HAV, 97.7% had no evidence of HBV, HCV or HIV; 1.7% had positive serology for HAV, HBV and HCV; 0.24% had positive serology for HBsAg, while 0.37% had positive serology for HCV. None of these cases were positive for HIV (Tables 2 and 3).

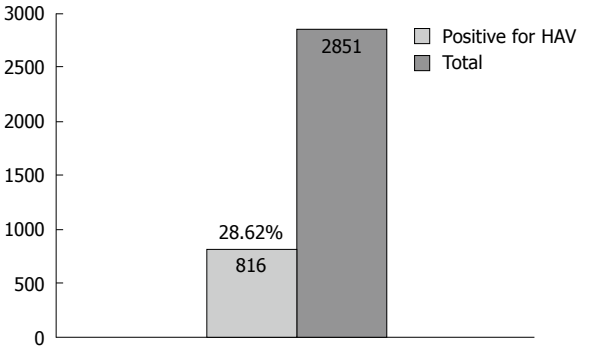

Figure 1 Patients with HAV+ve.

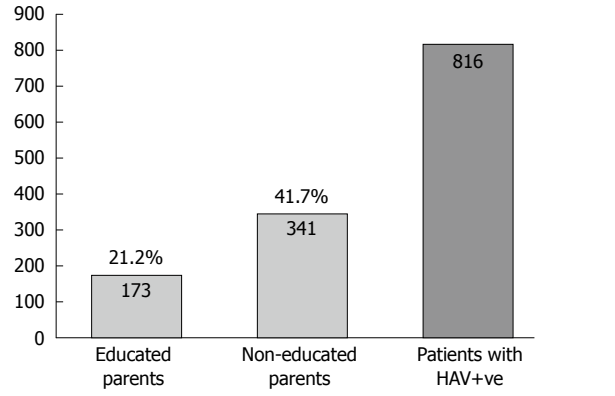

Figure 2 The prevalence of HAV among the group with non-educated parents or a single educated parent was higher than the educated group.

DISCUSSION

HAV is a non-enveloped, RNA-containing virus that belongs to the family Picornaviridae. It is a spherical 27-nm particle that was discovered by Feinstone in 1973[3]. The routes of infection are orofecal and percutaneous. The incubation period is about 28 d. The fecal shedding of virus is at a maximum during the late incubation period, just before or shortly after the onset of symptoms[4].

After oral inoculation of a chimpanzee with HAV, the viral antigen was detected first in the serum on day 14, in the tonsils on day 16 and in the liver on day 21. The viremia lasts for 2 wk[5]. In human studies, HAV RNA is detected for an average of 60 d after onset of clinical symptoms[6]. Risk factors that have been associated with reported HAV infection within the United States include sexual or household contact with another person with hepatitis (25%), contact with children attending a day-care center (15%), international travel (5%) and food or water-borne outbreak (5%). However, in 50% of cases, no risk factor can be identified[7].

In Kuwait, the epidemiology of HAV in the 1980s was similar to developing countries with almost 100% of

Table 1 Disease prevalence per age group *n* (%)

Age category	Pts with HAV+ve
Less than 27 yr (2385 cases screened)	577 (24)
28 to 40 yr (420 cases screened)	213 (51)
41 to 60 yr (46 cases screened)	26 (56.5)

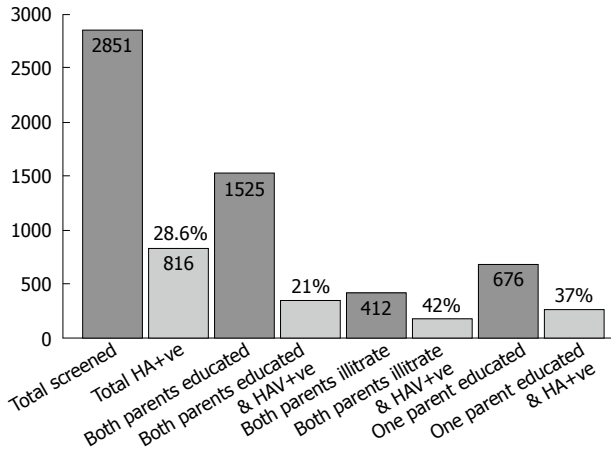

Figure 3 HAV and level education.

adults over the age of 20 years testing positive for anti-HAV. At that time, 90% of the screened cases with acute hepatitis A were below the age of 10 years and 70% below the age of 5 years[2]. Retrospective analysis of all charts of Kuwaiti patients presenting with acute HAV infectious between the ages of 0 and 15 years admitted to an infection disease hospital between 2000 and 2002 showed an incidence of 47 per 100 000. One third of these cases were from Jahra region, which is dominated by the Bedouin who live in large extended families. However, in 50% of these cases, there was no identified risk factor. Prolonged jaundice was found in 3% of cases and FHF in 0.4%[8].

There has been a dramatic drop in the incidence of HAV infection in children (47/100 000 *vs* 122/100 000) and a shift toward infection among older children (from 0-4 years to 7-12 years). Our study provides the most recent data about the prevalence of HAV in Kuwait over the last 20 years. The prevalence of HAV was 28% and one quarter of screened individuals below the age of 27 years had positive anti HAV. There were more cases in the areas dominated by Bedouin with extended families. Also, there were more cases among the group with uneducated parents, which reflects the relationship of the disease to low social background. These data show the shifting epidemiology of HAV in Kuwait toward intermediate to low endemicity, leaving 75% of the population below the age of 27 years non-immune, with a risk of exposure to HAV infection at a later age group, with increased morbidity. These changes have resulted from improvement in living standards and socio-economic progress. These data demonstrate the requirement of initiating an HAV vaccine program in Kuwait.

Areas in the Middle East like Qatar, United Arab Emirates and Saudi Arabia show a shifting pattern from high to intermediate endemicity for HAV. In Saudi Arabia, there are existing pockets of high HAV endemicity that may lead to outbreaks[9]. A study by Fathalla *et al*[10] showed that eastern Saudi Arabia still belongs to epidemiological pattern 1, which is characteristic of developing poor countries with low socioeconomic status and the country has a seroprevalence of 99%. The United Arab Emirates data showed that the seroprevalence of HAV was 60% and 90% for the ages of 16 and 40 years respectively which indicates a shift of HAV epidemic with infection towards an adult population[11]. In South-East Asia and China, there is shifting epidemiology of HAV from high to moderate or low endemicity. In China, this is associated with risk of outbreaks as a result of re-introduction of the virus from areas of high endemicity to low endemicity within a non-immune population[12] The incidence of HAV infection varies from high, moderate, low and very low endemicity areas. South-East Asia, India, Africa and Latin America were considered high endemic areas. The epidemiology of HAV is changing due to improvement in water supplies and sanitation conditions. Asian studies from Taiwan and India also show changing seroepidemiology of HAV infection and these countries are considering the use of HAV vaccine. The prevalence of HAV antibodies in Taiwan decreased in 1998 compared to 1992 reflecting the improvement of socioeconomic status and modernization of sanitation[13,14]. Africa is still considered as an area with high endemicity for HAV[9]. In Latin America, the highest anti-HAV seroprevalence rates were found in Mexico and the Dominican Republic. In these countries over the last 15 years, there was a shift towards medium endemicity with the peak of infection occurring in later childhood and adolescence rather than in early childhood. Contaminated water and food supply were the strongest risk factors in Latin America[15].

In European industrialized countries like Italy, there is a markedly lower prevalence of HAV infection,

Table 2 Association with other viral hepatitis

Anti HAV	HBsAg	Anti HCV	Count
Yes	Yes	Yes	14
Yes	Yes	No	2
Yes	No	Yes	3
Yes	No	No	797
No	Yes	No	97
No	No	Yes	30
No	No	No	1908

Table 3 Hepatitis A positive patients with HB, HC and HIV

Anti HAV+ve	HBsAg+ve	Anti HCV+ve	Anti HIV+ve	Number of cases
Yes	Yes	Yes	No	14
Yes	Yes	No	No	2
Yes	No	Yes	No	3
Yes	No	No	No	797

especially among a younger age group, due to marked improvements in socioeconomic conditions and hygienic standards. In the same region, small outbreaks of HAV infection were associated with intravenous drug abusers travelling to endemic areas, shellfish consumption and with an increasing number of family members[16].

Patients with negative serology for HAV need HAV vaccine. Also, patients with chronic liver disease who are non-immune to HAV need HAV vaccine[17]. In our study, 2035 (71%) of cases were not immune to HAV, 4.8% of them had positive HBsAg serology, while 1.5% of them had positive anti-HCV serology.

HAV vaccines are highly purified and formalin-inactivated. The vaccine was shown to be safe and effective when tested by Werzberger *et al*[18] among seronegative children 2-16 years of age in Monroe in 1992. Inactivated HAV vaccine (VAQTA, Merck and Co Inc, West Point, PA, USA) is given in two doses (0 and 6-12 mo). The estimated protective efficacy of one or more doses of the vaccine is 98%. HAV vaccine provides long-term immunity lasting probably from 20 to 50 years[19]. The vaccine has been available in the USA since 1995, and is highly effective in preventing disease transmission in a community with recurrent epidemics. The adverse effects of the vaccine are mild and include fever, rash and injection site reaction. It proved safe with no adverse effects among 30 000 vaccine recipients[20]. The HAV vaccine VAQTA given in two doses to a group of infants at the age of 2 years and followed up for 9 years provided long-term protection. It was effective in preventing HAV epidemics in the community in spite of the exposure to sporadic cases in non-vaccinated individuals[21]. HAV vaccine is recommended for persons with increased risk of infection including international travellers, illegal drug users, persons with chronic liver disease, persons who have clotting factor disorders and homosexuals[17]. Vaccination against hepatitis is the most effective means of preventing sexual transmission of hepatitis A and B[22].

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BRIEF ARTICLES

Iron homeostasis and H63D mutations in alcoholics with and without liver disease

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Abstract

AIM: To evaluate the prevalence of *HFE* gene mutation and indices of disturbed iron homeostasis in alcoholics with and without liver disease.

METHODS: One hundred and fifty-three heavy drinkers (defined as alcohol consumption > 80 g/d for at least 5 years) were included in the study. These comprised 78 patients with liver disease [liver disease alcoholics (LDA)] in whom the presence of liver disease was confirmed by liver biopsy or clinical evidence of hepatic decompensation, and 75 subjects with no evidence of liver disease, determined by normal liver tests on two occasions [non-liver disease alcoholics (NLDA)], were consecutively enrolled. Serum markers of iron status and HFE C282Y and H63D mutations were determined. *HFE* genotyping was compared with data obtained in healthy blood donors from the same geographical area.

RESULTS: Gender ratio was similar in both study groups. LDA patients were older than NLDA patients (52 ± 10 years *vs* 48 ± 11 years, $P = 0.03$). One third and one fifth of the study population had serum transferrin saturation (TS) greater than 45% and 60% respectively. Serum iron levels were similar in both groups. However, LDA patients had higher TS (51 ± 27 *vs* 36 ± 13, $P < 0.001$) and ferritin levels (559 ± 607 ng/mL *vs* 159 ± 122 ng/mL, $P < 0.001$), and lower total iron binding capacity (TIBC) (241 ± 88 μg/dL *vs* 279 ± 40 μg/dL, $P = 0.001$). The odds ratio for having liver disease with TS greater than 45% was 2.20 (95% confidence interval (CI): 1.37-3.54). There was no difference in C282Y allelic frequency between the two groups. However, H63D was more frequent in LDA patients (0.25 *vs* 0.16, $P = 0.03$). LDA patients had a greater probability of carrying at least one *HFE* mutation than NLDA patients (49.5% *vs* 31.6%, $P = 0.02$). The odds ratio for LDA in patients with H63D mutation was 1.57 (95% CI: 1.02-2.40).

CONCLUSION: The present study confirms the presence of iron overload in alcoholics, which was more severe in the subset of subjects with liver disease, in parallel with an increased frequency of H63D *HFE* mutation.

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Key words: Alcoholic liver disease; Iron; *HFE* gene; H63D; Hemochromatosis

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INTRODUCTION

Alcohol consumption and iron overload have long been found to be associated with each other. In 1896, the condition we now recognize as hereditary hemochromatosis was considered a variant of alcoholic cirrhosis[1], and even in the 1960s was believed to be a nutritional disorder related to alcohol intake, in which excess iron originated from the diet and iron content in

red wine[2].

Alcohol may increase iron absorption and cellular iron uptake by several possible mechanisms: (1) increased absorption *via* a non-carrier-mediated paracellular route[3]; (2) iron absorption is stimulated by anemia secondary to ineffective erythropoiesis due to alcohol-induced folic acid deficiency[4,5]; and (3) alcohol consumption is associated with decrease in enterocyte turnover through mitosis inhibition[6], which may reduce the already limited intestinal iron excretion. Recently, it has been shown that alcohol down-regulates hepcidin transcription, which leads to increased duodenal iron absorption *via* a divalent metal transporter-1 (with enhanced luminal import) and ferroportin protein expression (with enhanced basolateral translocation to the circulation)[7,8]. Furthermore, it has been shown that alcohol abolishes the iron-induced up-regulation of both liver hepcidin transcription and the DNA-binding activity of C/EBP alpha[9], thus negating the protective effect of hepcidin.

Suzuki *et al*[10] also demonstrated, an up-regulation of transferrin receptor expression in the hepatocytes of liver disease alcoholics (LDAs), which may promote hepatocyte iron accumulation.

Alcoholic liver disease (ALD) is often associated with elevated serum iron indices and hepatic iron overload[11-14]. Iron is also believed to be central in the pathogenesis of ALD, and some reports show iron overload as a predictive indicator of higher mortality[15], and development of hepatocellular carcinoma[16]. In fact, iron overload and alcohol have a synergistic effect on the production of oxidative stress[17-20].

The fact that only a minority of alcohol abusers, develop advanced liver disease such as steatohepatitis, fibrosis, and cirrhosis, prompted the search for genetic predisposing factors[21], such as C282Y and H63D mutations in the hemochromatosis protein HFE, which increases iron overload. However, no association has been found between C282Y *HFE* gene mutation and ALD, and there are conflicting reports on the association between H63D and ALD[22-27]. On the other hand, it is clear that the phenotypic expression of HFE C282Y homozygosity (the prototype for the genetic hemochromatosis syndrome) is low, and it increases markedly in patients with excessive alcohol consumption[28-30], which suggests that alcohol may act as a potential modifier of the (genetically determined) hemochromatosis phenotype.

The aim of the present study was to evaluate the prevalence of *HFE* mutations, and indices of disturbed iron homeostasis in alcoholics with and without liver disease.

MATERIALS AND METHODS

The study was approved by the Institutional Ethics Committees and written informed consent was obtained from the study subjects. A total of 284 heavy drinkers, defined as alcohol consumption > 80 g/d for at least five years were included in the study. The subjects consisted of consecutive patients seen in the Liver Unit (ambulatory or hospitalized) of a University Hospital, with suspected ALD; and consecutive referrals to two Alcohol Addiction Units, with psychiatric alcohol dependency, and no previous suspicion of liver disease.

Lifetime alcohol intake was assessed in all subjects, using a semi-structured questionnaire. Subjects were excluded from the study if they had any of the following: serological evidence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections or autoimmune liver disease, histological evidence of other liver diseases, or "mild" abnormalities of liver tests (bilirubin, aminotransferases, alkaline phosphatase, less than twice the upper limit of normal) in the absence of clinical signs of liver disease. Drinkers without clinical manifestations, and with normal liver tests on one occasion were excluded if it was not possible to obtain a second blood sample for reconfirmation.

Based on the above mentioned criteria, the subjects were divided into two categories: LDA and non-liver disease alcoholics (NLDA). The criteria for inclusion in the LDA group were the presence of either laboratory/clinical evidence of hepatic decompensation (e.g. ascites, varices, and encephalopathy) or liver histology compatible with LDA of severity greater than steatosis. Percutaneous liver biopsy specimens were evaluated blindly according to standard procedures[31]; only 11 subjects underwent this procedure since in patients with evidence of hepatic decompensation, such as ascitis, encephalopathy, or signs of portal hypertension, liver biopsy was considered unnecessary. Inclusion criteria for NLDA consisted of lack of clinical signs of liver disease and normal liver tests on two occasions (aminotransferases, prothrombin time, albumin and bilirubin) with the exception of an isolated rise in γ-glutamyl transferase[32]; no liver biopsy was performed in this group as it was considered unethical.

Laboratory tests

After a 12-h overnight fast, blood samples were collected and biochemical tests were done on the same day by routine methods, in the central pathology laboratory. The tests included: aminotransferases, bilirubin, γ-glutamyl transpeptidase, protein electrophoresis, prothrombin time, renal functions, cholesterol, triglycerides, ceruloplasmin, α-1 antitrypsin, anti-nuclear, anti-mitochondrial, anti-smooth muscle antibodies, and serological markers of HBV and HCV infections. Serum iron indices: iron, ferritin, total iron binding capacity (TIBC) and % transferrin saturation were also determined.

Genotyping

To detect the C282Y and H63D mutations, genomic DNA, extracted from the buffy coat fraction of whole blood was amplified by polymerase chain reaction as previously described[29,33]. The C282Y mutation creates a new *Rsa*I restriction site and the H63D mutation abolishes a *Mbo*I site allowing identification by restriction enzyme digestion.

A sub-group of 11 patients, all belonging to

Table 1 Clinical and laboratory characteristics of LDA and NLDA

	LDA (n = 78)	NLDA (n = 75)	P value
Age (yr)	52.3 ± 10.1	48.5 ± 10.7	0.03
Number of men (%)	66 (85)	62 (83)	NS
Alcohol consumption (g/d)	217 ± 195	327 ± 311	0.004
Presence of ascitis (%)	38 (52.1)	-	-
Presence of encephalopathy (%)	17 (34.7)	-	-
Alanine aminotransferase (r.v. 0-37 IU/L)	53 ± 60	17 ± 6	< 0.05
Aspartate aminotransferase (r.v. 0-41 IU/L)	81 ± 128	15 ± 5	< 0.05
Alkaline phosphatase (r.v. 40-129 IU/L)	143 ± 75	91 ± 21	< 0.05
γ-Glutamil transpeptidase (r.v. 8-61 IU/L)	225 ± 239	47 ± 48	< 0.05
Albumin (g/L)	36 ± 8	43 ± 3	< 0.05
Bilirubin (mg/dL)	4.0 ± 7.2	0.7 ± 0.9	< 0.05
Prothrombin time (seconds prolonged from control)	3.2 ± 2.7	0.5 ± 1.2	< 0.05
Cholesterol (mg/dL)	160.0 ± 84	209.1 ± 41.7	< 0.05
Triglycerides (mg/dL)	125.6 ± 111.3	162.8 ± 121.0	NS
Glucose (mg/dL)	119.9 ± 40.5	97 ± 13.6	NS
Iron (r.v. 65-175 μg/dL)	115 ± 64	99.4 ± 39	NS
TIBC (r.v. 250-425 μg/dL)	241 ± 88	279 ± 40	0.001
Transferrin saturation (%)	51 ± 27	36 ± 13	< 0.001
Ferritin (r.v. 23-236 ng/mL)	559 ± 607	159 ± 122	< 0.001

r.v.: Reference value; TIBC: Total iron binding capacity.

the LDA, had a liver biopsy; the degree of hepatic parenchymal siderosis was identified by Perl's iron stain, and graded from 0 to 4.

Statistical analysis

Basic descriptive statistics, means, standard deviation (SD), ranges and percentages, were used to characterize the populations. Categorical variables were analyzed by chi squared test and paired parametric numerical variables were compared, using the Student's t test. Correlations between several variables were evaluated through Spearman correlation coefficient.

Odds ratio analysis was used to explore interactions between iron overload and genetic mutations in the pathogenesis of ALD *vs* non-liver disease: the odds and 95% confidence intervals of having LDA outcome *vs* NLDA outcome were determined. LDA and NLDA were always the dependent variables and transferrin saturation > 45% or the presence of genetic mutations were evaluated as risk factors. All analyses were adjusted for patient's age.

The computer software used was Statistical Program for Social Sciences (SPSS) for Windows 12.0 (SPSS Inc., Chicago, USA, 2004). All P values were two-sided; for all statistics, significance was accepted at the 5% probability level.

RESULTS

Based on the predefined inclusion and exclusion criteria, 153 heavy drinkers were included, 78 in the LDA group and 75 in the NLDA group. Clinical and biochemical characteristics of the study groups are summarized in Table 1. The gender ratio was similar in both groups; LDA patients were older (52.3 ± 10.1 years *vs* 48.5 ± 10.7 years, P = 0.03); and alcohol consumption was lower in LDA compared to NLDA (217 ± 195 g/d *vs* 327 ± 311 g/d, P = 0.004).

Both groups had similar mean iron concentrations (Table 1), however, LDA patients had lower TIBC (241 ± 88 μg/dL *vs* 279 ± 40 μg/dL, P = 0.001), and higher levels of ferritin (559 ± 607 ng/mL *vs* 159 ± 122 ng/mL, P < 0.001) and serum transferrin saturation (51% *vs* 36%, P < 0.001). Overall, among the 153 heavy drinkers, 33% had serum transferrin saturation greater than 45%, while 20% had greater than 60%; transferrin saturation higher than 45% and higher than 60% were more frequent in LDA patients (47.4% *vs* 18.1%, P < 0.001, and 34.6% *vs* 5.3%, P < 0.001, respectively). Furthermore, in subjects with transferrin saturation higher than 45%, the odds ratio for having LDA was 3.90 (95% confidence interval (CI): 1.59-4.54, P < 0.0001).

In the 11 patients who had a liver biopsy, there was a significant association between serum ferritin levels and the degree of hepatic parenchymal siderosis, as identified by Perl's iron stain (r = 0.692, P = 0.02). Five of seven patients (71%) with Perl's staining > 1, had H63D mutation, compared with two of four (50%) in those with a score of 1 or less (r = 0.217, P = 0.547). The distribution of C282Y and H63D genotypes is shown in Table 2. Allelic frequency of H63D mutation was higher in LDA than in NLDA patients (0.25 *vs* 0.16, P = 0.032). Furthermore, allelic frequencies of H63D mutation in NLDA subjects were similar to that seen in the general population from the same geographical area, based on the data on healthy blood donors[34]. There were no differences in the allelic frequency of C282Y between the two groups.

The odds ratio of having LDA and H63D mutation was 1.75 (95% CI: 1.02-2.40, P < 0.03), while the odds ratio of carrying at least one *HFE* mutation was 1.56 (95% CI: 1.05-2.32, P < 0.03).

The serum transferrin saturation and ferritin levels were higher in subjects carrying at least one *HFE* mutation

Table 2 Comparison of *HFE* genotypes, with C282Y or H63D allelic frequencies in LDA *vs* NLDA subjects and a control population *n* (%)

	LDA (*n* = 78)	NLDA (*n* = 75)	Blood donors[34] (*n* = 133)
wt/wt	39 (50)	52 (68)	92 (69)
wt/H63D	31 (39.7)	19 (25.3)	27 (20)
wt/C282Y	3 (3.8)	2 (3.7)	7 (5)
H63D/H63D	3 (3.8)	2 (3.7)	6 (4)
C82Y/H63D	2 (2.5)	1 (1.3)	1 (1)
C282Y allelic frequency	0.032	0.02	0.03
H63D allelic frequency[1]	0.25	0.16	0.15
Any mutation[1]	39 (50)	24 (32)	41 (31)

wt: Wild type; [1]P = 0.02; There were no other statistically significant differences between the various groups.

compared with subjects without *HFE* mutation (49% ± 24% *vs* 39% ± 23%, P = 0.02 and 499 ± 600 ng/mL *vs* 258 ± 339 ng/mL, P = 0.005, respectively) (Table 3). Moreover, the presence of one H63D mutation in patients with transferrin saturation > 45% increased the odds ratio for having LDA to 2.17 (95% CI: 1.42-3.32, P < 0.01).

DISCUSSION

In the present study, heavy drinking was frequently associated with iron overload, as suggested by elevated serum ferritin levels and transferrin saturation, in the absence of hemochromatosis[35]. Moreover, iron overload was more intense in the presence of liver disease, as shown by higher serum concentrations of ferritin and transferrin saturation.

Although ferritin and transferrin saturation may be questioned as markers of iron overload in the presence of liver disease, since ferritin elevation may result from necroinflammatory activity, and decreased hepatic protein production may occur secondary to liver disease[36], resulting in lower TIBC and higher transferrin saturation, previous studies in patients with liver disease have shown significantly higher ferritin levels in patients with alcohol-related liver disease[12]. Furthermore, in the present study, we observed a positive correlation between serum ferritin and the degree of hepatic iron deposition in patients who had a liver biopsy.

Since iron plays an important pathological role in ALD[37], and alcoholics are more prone to develop iron overload, it is conceivable that alcoholics who tend to absorb and store more iron are at an increased risk of liver disease. The presence of mutations in the hemochromatosis *HFE* gene may serve as a predisposing factor for the development of liver disease. However, five previous studies failed to show a relationship between ALD and the presence of such mutations[22-26]. On the other hand, Ropero Gradilla *et al*[27], in Spain, observed an association between H63D mutation (but not with C282Y mutation) and the risk of advanced liver disease. In the present study, individuals carrying at least one *HFE* mutation had a significantly higher probability of having liver disease, which suggested an association between *HFE* mutation and increased susceptibility to ALD. However, it is possible that our observation of an increased prevalence of *HFE* mutations may be a casual finding (type I error).

Table 3 Serum iron indices according to *HFE* status

	At least one *HFE* mutation (*n* = 63)	No *HFE* mutation (*n* = 90)	*P* value
Iron (μg/dL) (r.v. 65-175)	118 ± 52	100 ± 55	NS
TIBC (μg/dL) (r.v. 250-425)	262 ± 83	259 ± 63	NS
Transferrin saturation (%)	49 ± 24	39 ± 22	0.02
Ferritin (ng/mL) (r.v. 23-236)	499 ± 600	258 ± 339	< 0.001

r.v.: Reference value; TIBC: Total iron binding capacity.

The extent to which H63D mutation predisposes to iron overload has been the subject of much debate. Such an association has been observed in homozygosity studies[38,39], and also with the findings that serum transferrin saturation is significantly increased in H63D homozygotes and heterozygotes as compared with wild-type individuals[40]. To reinforce the importance of the *HFE* mutations as risk factors for liver disease, the presence of these mutations should be associated with significantly higher iron parameters. Indeed, the present study showed that transferrin saturation and ferritin concentration were higher in patients with at least one *HFE* mutation, with no difference in the TIBC values. However, even in the sub-group of individuals with increased iron saturation, the presence of H63D mutation was associated with a higher probability of liver disease, suggesting that H63D mutation may be a risk factor independent of the associated iron overload.

In conclusion, the present study has confirmed previous reports of the presence of iron overload in alcoholics, which is more severe in the subset of subjects with liver disease, and is associated with an increased frequency of H63D *HFE* mutation. Our findings indicate that H63D *HFE* mutation, by further increasing iron overload, is a risk factor for liver disease, through the synergistic damaging effects of alcohol and iron. Further research is needed to evaluate if the progression of the liver disease in alcoholic patients with iron overload is associated with a worse prognosis.

COMMENTS

Background

Alcohol abuse enhances iron absorption and may play a crucial role in the pathogenesis of alcoholic liver disease (ALD). Thus, conditions that enhance iron uptake may have a synergistic role in the development of ALD. Currently, the relevance of hemochromatosis-associated gene mutations and/or iron status in ALD is unclear.

Innovations and breakthroughs

The fact that only a minority of alcohol abusers, develop advanced liver disease such as steatohepatitis, fibrosis, and cirrhosis, prompted the search for genetic predisposing factors, such as C282Y and H63D mutations in the hemochromatosis protein HFE, which increases iron overload. However, no association has been found between C282Y *HFE* gene mutation and ALD, and there are conflicting reports on the association between H63D and ALD. The aim of the present study was to evaluate the prevalence of *HFE* mutations,

and indices of disturbed iron homeostasis in alcoholics with and without liver disease.

Applications

The research reported that H63D HFE mutation, by further increasing iron overload, is a risk factor for liver disease, through the synergistic damaging effects of alcohol and iron.

Peer review

The paper is interesting and is focused on original topic. Title reflects the content of the article. Results and discussion provide accurate informations and lead to conclusions

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BRIEF ARTICLES

Use of albendazole sulfoxide, albendazole sulfone, and combined solutions as scolicidal agents on hydatid cysts (*in vitro* study)

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Abstract

AIM: To establish which scolicidal agents are superior and more suitable for regular use.

METHODS: *Echinococcus granulosus* protoscoleces were obtained from 25 patients with liver hydatid cysts. Various concentrations of albendazole sulfone, albendazole sulfoxide, and albendazole sulfone and albendazole sulfoxide mixed together in concentrations of 50 μg/mL, and H_2O_2 in a concentration of 4%, NaCl 20%, and 1.5% cetrimide-0.15% chlorhexidine (10% Savlon-Turkey) were used to determine the scolicidal effects. Albendazole (ABZ) derivatives and other scolicidal agents were applied to a minimum of 100 scoleces for 5 and 10 min. The degree of viability was calculated according to the number of living scolices per field from a total of 100 scolices observed under the microscope.

RESULTS: After 5 min, ABZ sulfone was 97.3% effective, ABZ sulfoxide was 98.4% effective, and the combined solution was 98.6% effective. When sulfone, sulfoxide and the combined solutions were compared, the combined solution seemed more effective than sulfone. However, there was no difference when the combined solution was compared with sulfoxide. After 10 min, hypertonic salt water, sulfone, sulfoxide, and the combined solution compared to other solutions looked more effective and this was statistically significant on an advanced level. When sulfone, sulfoxide, and the combined solutions were compared with each other, the combined solution appeared more effective than sulfone. When the combined solution was compared with sulfoxide, there was no difference.

CONCLUSION: Despite being active, ABZ metabolites did not provide a marked advantage over 20% hypertonic saline. According to these results, we think creating a newly improved and more active preparation is necessary for hydatid cyst treatment.

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Key words: Hydatid disease; Albendazole; *In vitro* study; Combined solution; Scolicidal agents

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Adas G, Arikan S, Kemik O, Oner A, Sahip N, Karatepe O. Use of albendazole sulfoxide, albendazole sulfone, and combined solutions as scolicidal agents on hydatid cysts (*in vitro* study). *World J Gastroenterol* 2009; 15(1): 112-116 Available from: URL: http://www.wjgnet.com/1007-9327/15/112.asp DOI: http://dx.doi.org/10.3748/wjg.15.112

INTRODUCTION

Treatments of hydatid disease have been suggested in many examples within the literature and text books, including systemic administration of various chemotherapeutic agents, surgery, and the percutaneous approach. The efficacy of systemic chemotherapy is limited[1-4]. Although surgery is the recommended treatment for liver hydatid cyst, percutaneous treatment has been introduced as an alternative treatment, especially in patients who cannot or do not want to undergo surgery[4,5]. There is no completely satisfactory surgical approach, but the operation is best performed by experts. The main object is to remove the cyst completely, without soiling and infecting the peritoneum and with complete obliteration of the resulting dead space. Complete removal of the cyst, with its adventitia, is ideal to avoid spilling the contents. The usual operation is cystectomy with removal of the germinal and laminated layers and preservation of the host derived

ectocyst[6]. Instillation of a scolicidal agent into hepatic hydatid cysts to reduce the risk of spillage of viable protoscolices is an integral part of the surgical technique for many surgeons. For over 20 years, benzoimidazole derivatives have been widely used in the medical treatment of hydatidosis. The current literature suggests that amongst all of these, albendazole (ABZ) is the most effective and useful drug for the medical treatment of hydatid disease[7]. After oral administration, ABZ is oxidized to a sulfoxide, which is in part further oxidized to a sulfone, and ABZ sulfoxide is the main metabolite *in vivo*[8]. The metabolites of ABZ are characterized by their low solubility in water and poor absorption[9]. ABZ has a scolicidal effect via its biologically active metabolites ABZ sulfoxide and ABZ sulfone, the latter being more effective. The first aim of this *in vitro* study was to establish the use of ABZ sulfoxide, ABZ sulfone, and the combined solution as scolicidal agents on the hydatid cyst. The second aim was to compare their effectiveness with other scolicidal agents.

MATERIALS AND METHODS

Protoscolex collection

For this *in vitro* study, we used scolex solutions collected from 25 patients who underwent operations for liver hydatid disease at the Haseki Teaching and Research Hospital, Department of General Surgery from 2002 to 2004. All samples were examined and identified at the Istanbul University, Faculty of Medicine, Department of Microbiology and Clinical Microbiology. For this study, ABZ sulfone and ABZ sulfoxide were purchased from Unimark Remedies Ltd Company in India (Certificate of Analysis of working standard ABZ/IMP001/02-Date 17.04.2002).

Effectiveness of ABZ and other solutions in vitro

ABZ sulfone and ABZ sulfoxide dissolve poorly and slowly in water; however, in order to avoid the effects of other solvents, normal saline (0.9% NaCl) was used as a solvent, so that any scolocidal effect and tissue injury would be due only to ABZ sulfone and ABZ sulfoxide. Preparation of the ABZ sulfone and ABZ sulfoxide solutions was as follows: 10 mg ABZ sulfone and ABZ sulfoxide was dissolved in normal saline and brought to a final volume of 100 mL, and then the solution was mixed for 12 h at room temperature by a magnetic mixer. The solutions were further diluted by normal saline to obtain 50 μg/mL ABZ sulfone and ABZ sulfoxide in the final working solutions. We diluted another solution of ABZ sulfone and ABZ sulfoxide mixed together to a total of 50 μg/mL. All three solutions were sterilized by UV. Moreover, for comparison, we used H_2O_2 in 4% concentration, NaCl 20%, and 1.5% cetrimide-0.15% chlorhexidine (10% Savlon-Turkey) solution to determine the scolicidal effects.

The scolices were mixed with each solution for 5 and 10 min. The scolices were separated from the solutions and washed by normal saline. A few drops were smeared on an object glass and a drop of eosin was added; the slide was then covered with a cover glass and evaluated under the light microscope. For each different solution at both 5 and 10 min, the percentages of dead scolices were determined by counting a minumum of 100 scolices. With regard to the viability of cysts, the same criteria were used as defined in a previous report[10]. Scolices that lost their ellipsoid shape and became round, had rostellums rolled in, showed vacuolar degeneration, and took in eosin were considered dead; all four criteria had to be met for a scolex to be accepted as dead.

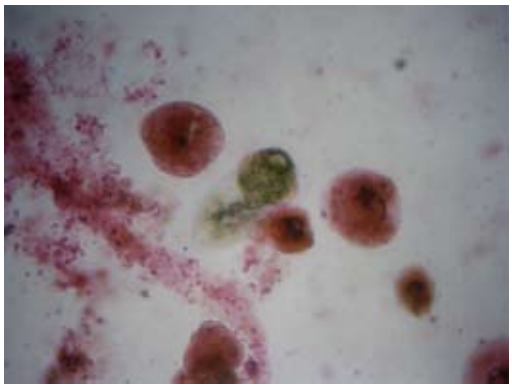

Figure 1 Light microscopy of *Echinococcus granulosus* microcysts incubated *in vitro* with ABZ.

Statistical analysis

All statistical calculations were based on an analysis of comparing means by a paired samples *t* test. Differences were determined using the least significant differences, and $P < 0.05$ was considered to be significiant.

RESULTS

This *in vitro* study demonstrated that 50 μg/mL ABZ sulfone killed 97.3% of the scolices, ABZ sulfoxide killed 98.4% of the scolices, and the combined solution (sulfone + sulfoxide) killed 98.6% of the scolices in 5 min (Figure 1). The second part of the study showed that the three ABZ solutions respectively killed 98.6%, 99.5%, and 99.6% of the scolices in 10 min. Among the other solutions that were used, hypertonic salt water was seen to be most effective on scolices. Hypertonic saline solution respectively killed 98.2% and 99.5% of the scolices in 5 and 10 min (Table 1). In the first part of the study, the scolicidal effects of hypertonic salt water, the ABZ sulfone, ABZ sulfoxide, and the combined solution were seen to be statistically significant on an advanced level compared to cetrimide and peroxide. When sulfone, sulfoxide, and the combined solution were compared to hypertonic salt water, no statistical significance was seen ($P > 0.05$). When sulfone, sulfoxide, and the combined solutions were compared with each other, the combined solution was seen to be more effective. When comparing the combined solution to sulfoxide there was not any difference.

In the second part of the study, again hypertonic salt water, sulfone, and the combined solution were seen to be more effective compared to other solutions and were

Table 1 The use of different scolicidal agents at the 5th and 10th min for the treatment of hydatid disease (*in vitro* results)

Time	NaCl 20%	H_2O_2	Cetrimide	Sulfone	Sulfoxide	Sulfone + Sulfoxide
5th min	98.2%	90.3%	86.9%	97.3%	98.4%	98.6%
10th min	99.5%	95.7%	92.6%	98.6%	99.5%	99.6%

Table 2 Different scolicidal agents in literature

Author	Agents	Results
Caglar *et al*[26] (2008)	20% silver nitra (20 min)	100% death
	50% Dextroz (30 min)	100% death
	20% NaCl (45 min)	100% death
	20% Mannitol (45 min)	100% death
Frayha *et al*[27] (1981)	Cetrimide 0.5%-1% (10 min)	100% death
Kayaalp *et al*[28] (2002)	10%-30% NaCl (3, 6, 75 min)	100% death
Sonişik *et al*[11] (2002)	10%-30% NaCl (3, 6 and 75 min)	100% death
Besim *et al*[16] (1989)	20% Saline (15 min)	100% death
	95% Ethyl alcohol (15 min)	100% death
	10% Polyvinyl pirrolidone iodine (15 min)	100% death
	3% H_2O_2 (15 min)	100% death
Erzurumlu *et al*[20] (1998)	Albendazole sulfoxide 20 μg/mL	5% death
	Albendazole sulfoxide 50 μg/mL	50% death
	Albendazole sulfoxide 100 μg/mL	100% death
Adas *et al* (our study) (2008)	20% NaCL (5 min)	98.2% death
	3% H_2O_2 (5 min)	90.3% death
	Cetrimide (5 min)	86.9% death
	Albendazole sulfone (5 min)	97.3% death

seen to be statistically significant on an advanced level as well. When sulfone, sulfoxide and the combined solution were compared to hypertonic salt water, hypertonic salt water was seen to be statistically significant compared to sulfone, but it was not seen to be significant compared to the others. When sulfone, sulfoxide and the combined solution were compared to each other, the combined solution was seen to be more effective compared to sulfone. Upon comparing the combined solution with sulfoxide, there was not any difference.

When each solution was compared based on 5 and 10 min timing in the first and the second part of the study, the scolicidal effects of each solution in the second part were statistically significant ($P < 0.05$).

DISCUSSION

There are currently three treatment options for hydatid disease of the liver: surgery, which remains the most efficent treatment, percutaneous aspiration and medical treatment. In general, hydatidosis is a public health problem, especially in our country, and the treatment often is selected depending on the social and medical professional's conditions. Since the 1990s, percutaneous treatment has been used increasingly. Surgery remains the most effective treatment, which aims to treat concurrently the parasitic disease, the cavity, and often the biliary complications. Although surgery is technically demanding and often considered risky, the development of hepatic surgery permits safer performance of this therapeutic option.

Ideally, therapy of liver hydatid disease should be able to cure the disease with a low morbidity. Failure of treatment is defined as recurrence and complications related to the intervention. Protection of the operation field is mandatory before the planned operation on the cyst or before the cyst is emptied. Preoperative destruction of the cyst's contents and preventing infection of the surrounding area has an important role for success of the operation; also, this procedure helps to prevent the illness from returning[11]. For sterilization of the cyst, several parasiticidal substances have been used. Scolicidal solutions remain indispensable in the treatment of hydatid cyst disease. Properties of an ideal solution would be inexpensiveness and the promotion of a rapid and complete scolicidal effect with an absence of local and systemic side effects. From this point of view, no ideal solution and agents have been described yet.

ABZ carbamate is one of the most important benzimidazole derivatives used against liver flukes, tapeworms, and lung and gastrointestinal roundworms. ABZ is normally not detectable in human plasma since it is rapidly metabolized to its major active metabolite as ABZ sulfoxide and ABZ sulfone. ABZ sulfoxide is the main metabolite *in vivo*[8,12-14]. ABZ leads to a decrease in the glycogen content of the cyst wall, inhibits acid phosphatase, ATP, pyruvate kinase, phosphoenolpyruvate kinase, and causes cellular autolysis and degeneration in the microthrics and the microtubuli[15].

Despite ABZ being used preoperatively and postoperatively for hydatid cyst treatment, today there is no comparative study in the literature, especially concerning ABZ metabolites' preoperative scolicidal effectiveness (Table 2). In our study, the ABZ metabolites of ABZ sulfoxide, ABZ sulfone, and the combined form were applied directly on the scolices. In this case, the reason why we chose ABZ metabolites over ABZ was that ABZ was not effective directly on the body. Also, we determined how much time was needed for each of the metabolites to be effective and on how many scolices they were effective.

In both parts of the study, cetrimide was found to be the least effective in killing the scolices. This result showed a dissimilarity from other limited studies performed in the literature. Besim *et al*[16] reported that cetrimide-chlorhexidine was the most potent scolicidal agent *in vitro*. Half-percent cetrimide and 0.05% chlorhexidine combination for 5 min is an effective protoscolicidal agent in labaratory and clinical

studies[16]. Sonişik *et al*[11] further supported the potent scolicidal effect of cetrimide with a clinical study. The disadvantages of cetrimide, namely metabolic acidosis and methemoglobinemia, have been reported[17]. In the literature, hypertonic salt water (20%) was 100% effective on scolices after 6 min, and this is probably the most widely used scolicidal agent in current practice because of its availability and effective scolicidal properties[17]. In our study, this treatment was found to be 98.2% effective at the end of min and 99.5% effective at the end of 10 min[17]. Hypertonic saline can cause acute hypernatremia. H_2O_2 was not found to be 100% effective on scolices in our study. Because of the side effects, it is not used in many fields today. Landa García *et al*[18] tested four scolicidal agents (10% H_2O_2, 10% providone iodine, praziquantel, 10% hypertonic saline) and reported that 10% H_2O_2 and 10% providone iodine were much more potent than the other agents. Even though 10% H_2O_2 was defined as a powerful scolicidal agent *in vitro* by Meymerian *et al*[19], fatal air embolism and anaphylactic shock have also been reported with 10% H_2O_2.

Today, ABZ and its metabolites are not used as scolicidals for the preoperative routine. ABZ is the most widely used substance for the medical treatment of hydatid cyst disease. It has a scolicidal effect via its biologically active metabolites sulfone and sulfoxide. ABZ sulfoxide is more effective than ABZ sulfone. There is no study examining whether one of the metabolites or combinations are more effective as a preoperative scolicidal. On this topic, there have been two experimental studies by Erzurumlu *et al*[20-22]. He demonstrated that 20 μg/mL ABZ sulfoxide killed 5% of the scolices in 15 min, scolicidal activity was 50% with a 50 μg/mL solution, and it was 100% for a 100 μg/mL solution[20]. The other study showed that 10%, 5% and 1% ABZ solutions had complete scolicidal effects[21]. ABZ was used experimentally for percutaneous treatment of hydatid cysts. Deger *et al*[3] demonstrated that ABZ sulfoxide injection as a scolicidal agent in the percutaneous treatment of cystic echinococcosis seems to be effective in sheep. Yetim *et al*[22] further supported percutaneous treatment with the idea that ABZ solutions are effective as scolicidal solutions on rabbits. Greater scolicidal effects and fewer side effects on the hepatobiliary system are the advantages of ABZ solution[22]. It is known that scolicidal solution injection in the cysts or the biliary system leads to a rise in liver enzyme level. It has also been shown that systemically administered ABZ can lead to the same changes[3,22,23].

When the first and the second part of the study were compared, it was seen that all of the solutions that were used were more effective at the end of 10 min. This result was statistically significant, as it showed us that 5 min of preoperative waiting time was not enough.

In conclusion, none of the solutions killed 100% of the scolices at the end of 5 and 10 min. With the aim of preventing the spread to the operations' surrounding area and the peritoneum, it is necessary to strictly protect the surrounding area of the cyst. Despite the effectiveness of ABZ metabolites, no distinctive advantage was provided compared with 20% hypertonic saline. Its harder obtainability, harder preparation, and high cost are some of the important disadvantages. For this reason, it cannot be seen as an ideal scolicidal agent. An ideal scolicidal agent is defined as being potent in low concentrations, acting in a short period time, being stable in cyst fluid, not affected by dilution with the cyst fluid, being able to kill the scolex in the cyst, being non-toxic, having low viscosity, and being readily avaliable and easily prepared, as well as being inexpensive[24,25]. In conjunction with prevention of cystic fluid spillage, total evacuation and prevention of any contact of the germinative membrane with the peritoneal surface are essential because the germinative membrane can contain viable protoscoleces despite proper cyst fluid inactivation. Walling off the surgical field with laparotomy sponges soaked in scolicidal agents is the most important step in hydatid cyst surgery. If ABZ metabolites can be obtained for commercial use and can be inexpensive, the usage of them could be more common. Combined and sulfoxide solutions were found to be more effective than sulfone. In our clinical practice, it is usually the 20% hypertonic saline solution that is used. However, it presents serious intraoperative problems related to hypernatremia. Therefore, we need less harmful but more effective drugs in hydatid disease treatment. Our study sheds light on the fact that ABZ metabolites may well meet this requirement.

COMMENTS

Background

Using scolicidal agents is of great importance in hydatid disease. The aim of this study was to find the most effective and appropriate scolicidal agents.

Research frontiers

Albendazole (ABZ) metabolites are highly effective as scolicidal agents. When compared with each other, combined and sulfoxide solutions were found to be more effective than sulfone. When it comes to the comparison of ABZ metabolites with 20% hypertonic saline, no distinctive advantage was provided

Applications

ABZ metabolites, the most effective scolicidal agents, hydatid disease treatment, the comparison of various scolicidal agents.

Terminology

Hydatid disease is a potentially fatal parasitic disease that can affect many animals, including wildlife, commercial livestock and humans. ABZ is a broad spectrum anti-protozoal and anti-helminthic compound used as a drug indicated for the treatment of a variety of worm infestations. ABZ metabolites include sulfone, sulfoxide, and the combined solution.

Peer review

The manuscript deals with the topic with adequate methods and conclusive results. It's very interesting.

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CASE REPORT

An unusual presentation of sclerosing mesenteritis as pneumoperitoneum: Case report with a review of the literature

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Abstract

Sclerosing mesenteritis is a rare condition that involves the small or large bowel mesentery. An unusual presentation of this condition, which led to difficult preoperative assessment and diagnosis, is described. This report is followed by a comprehensive review of the literature.

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Key words: Mesenteritis; Panniculitis; Small bowel obstruction; Mesentery

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INTRODUCTION

Sclerosing mesenteritis is a rare benign condition that affects the intestinal mesentery. Its presentation can be quite varied because of the different histopathological processes involved in the evolution of this condition. Diagnosis can be challenging, as is its management. An unusual presentation of this condition is described, followed by a comprehensive review of the literature.

CASE REPORT

A 74-year-old man was admitted as an emergency with acute abdominal pain and distension. He previously underwent an abdominoperineal resection 10 years ago for rectal cancer, and most recently had a surveillance colonoscopy about 2 wk prior to admission.

On initial assessment, he had a slightly elevated temperature (37.4℃). There were no signs of peritonitis, but his abdomen was slightly distended and had minimal central abdominal tenderness. His blood investigations, which included a full blood count, C-reactive protein, amylase, serum electrolytes and liver function tests, were all within normal limits. The erect chest X-ray showed pneumoperitoneum on both sides, and the abdominal film showed some distended small bowel loops in the central abdomen.

Although he had evidence of a perforated viscus on the X-ray, he was managed conservatively, in view of his relative clinical wellbeing and lack of hematological and biochemical abnormalities. He was kept nil by mouth, had a nasogastric tube inserted, commenced on intravenous fluids and broad-spectrum antibiotics, and was kept under close observation.

An upper gastrointestinal perforation was excluded by means of a Gastrograffin meal. Over the course of the next few days, his clinical condition improved with conservative management. However, his repeat chest films continued to show free gas under both his diaphragms, and we arranged for a computerized tomography (CT) scan of his abdomen and pelvis, with oral and intravenous contrast. This again confirmed the pneumoperitoneum and a few dilated small bowel loops. There was no extravasation of the contrast material into the peritoneal cavity (both small and large bowel loops

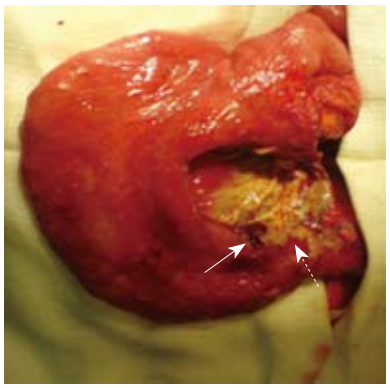

Figure 1 Intraoperative findings, showing small bowel obstruction from an area of localized thickening (dashed arrow), with a proximal perforation (arrow). The proximal small bowel is dilated and thickened, suggesting chronic obstruction.

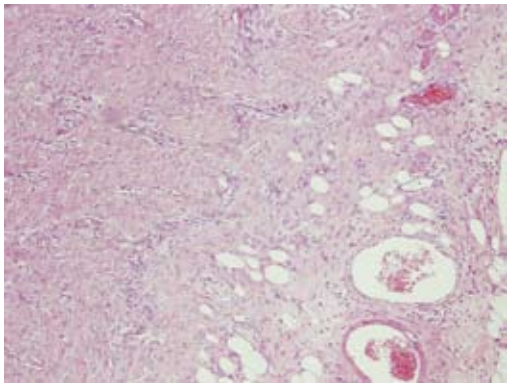

Figure 2 Histology of the specimen. Section through the mesentery, showing fibrocollagenous stroma with dilated vessels.

were filled with the contrast agent) or any obvious point of obstruction.

On day 8 following his admission, he started vomiting feculant material, which prompted an urgent midline laparotomy. At operation, there was a small amount of turbid fluid in the peritoneal cavity and an area of near complete obstruction at the mid-part of the small bowel. At this location, there was also a localized collection of small bowel contents. Part of the wall of this collection was made up of the adjacent collapsed loops of small bowel and mesentery. On dissecting this area, the point of obstruction was a 2-3-cm area of circumferentially thickened small bowel, and there was a tiny perforation just proximal to this (Figure 1). Adjacent mesentery was thickened. The proximal bowel wall showed signs of chronic obstruction. The previous colostomy in the left iliac fossa was normal and there was no evidence of any recurrent cancer. As the intraoperative finding was suspicious of a primary small bowel malignancy, nearly 40 cm of the small bowel was resected with wide mesenteric clearance, and a side-to-side hand-sewn jejuno-jejunal anastomosis was carried in two layers.

Postoperatively, the patient developed several complications, such as bilateral pneumonia, adult respiratory distress syndrome, paralytic ileus and renal failure. He unfortunately succumbed to these complications and died after a prolonged stay in the critical care unit.

The histopathological examination of the narrowed segment of the small bowel showed concentric fibrosis of the mesentery, serosa and outer part of the muscularis propria, with a slightly edematous mucosa. The fibrous tissue was arranged in lobules and was composed of fibroblast-like spindle cells arranged in a fascicular pattern. Sections through the thickened mesentery showed marked fibrocollagenous thickening, without fat necrosis or inflammation (Figure 2). No granulomas or foreign-body reaction were seen. The perforated area showed fibrinopurulent exudates, with no ulcers or other lesions. Immunohistochemistry of the young and collagenized fibrous tissue was positive for smooth muscle actin, vimentin and cytokeratin but negative for desmin, S100, CD117 and CD34. The overall appearance was that of fibrosing mesenteritis.

Table 1 Various nomenclature used to describe this condition[3,5-7]

Sclerosing mesenteritis[1]	Xanthogranulomatous mesenteritis
Retractile mesenteritis	Mesenteric lipogranuloma
Liposclerotic mesenteritis	Systemic nodular panniculitis
Mesenteric weber-christian disease	

[1]Preferred terminology.

Table 2 Association with other idiopathic inflammatory disorders[1,3,4,7-9]

Retroperitoneal fibrosis	Sclerosing cholangitis
Riedel thyroiditis	Sclerosing mediastinitis
Orbital pseudotumor	Desmoplastic metastatic carcinoma
Whipple disease	Sarcoma
Mesenteric fibromatosis (intra-abdominal desmoid tumour)	

DISCUSSION

Sclerosing mesenteritis is a rare, idiopathic and benign disease of the abdominal mesentery first described by Jura in 1924[1-3]. It is defined as a mass that is found in the lining of the mesentery[4]. Currently, just over 300 cases have been published to date, with the majority being case reports and the rest being very small series.

In addition to sclerosing mesenteritis, other nomenclature has been used (Table 1). In histolo-pathological terms, the preferred terminology is sclerosing mesenteritis[7]. The different terminology used represents the different histological features found, despite the clinical entity being the same. This disorder has been linked with other idiopathic inflammatory disorders (Table 2).

The vast majority of cases of sclerosing mesenteritis are considered as idiopathic. Autoimmunity, ischemia, infection, vasculitis, Gardner's syndrome, carcinoid, trauma and previous abdominal surgery have all been linked[1,3,9-11]. In the series of Daskalogiannaki *et al*[12], sclerosing mesenteritis was related to malignancy in 69% of patients. The association with malignancy may be coincidental or secondary to an autoimmune inflammatory reaction, the mechanism of which has not yet been elucidated. These malignancies included

Table 3 Different stages of disease and treatment[1-3,5,7,13,15]

Stage of disease	Predominant histological feature	Preferred terminology	Treatment
Early stages	Fat necrosis	Mesenteric lipodystrophy	None
Intermediate stages	Inflammation	Mesenteric panniculitis	Immunosuppressants[18,19] Colchicine[3,20] Tamoxifen[2] Corticosteroids[3,19,20] Progesterones[2,21]
Final stages	Fibrosis	Sclerosing or retractile mesenteritis	Surgery (where indicated)

lymphoma, breast cancer, lung cancer, melanoma and colon cancer. In our case, previous surgery for rectal cancer may have been a causative factor, despite the fact that the surgery was a decade ago.

Mean age at presentation of sclerosing mesenteritis is in the fifth and sixth decades, and it is seen twice as frequently in males than females[1,8,13]. The disease can present clinically as single or multiple masses, or as a diffuse thickening of the mesentery[1,2,7,8,13]. The most common site of this disease is the small bowel mesentery. However, sole involvement of the mesocolon (the most common site being the rectosigmoid colon)[9] has been found in 20% of cases. Rare sites described are the mesoappendix, peripancreatic area, omentum and pelvis[4,7,9].

Clinical manifestations of this entity are non-specific. Patients may present asymptomatically with an incidental diagnosis. The various clinical features include abdominal pain, vomiting, diarrhea, constipation, anorexia, weight loss, fatigue, fever of unknown origin, ascites, pleural and pericardial effusion[2-4,7-9,13,14]. In our case, the presentation was complex, with the patient presenting with pneumoperitoenum following a recent colonoscopy, which suggested an iatrogenic complication. In retrospect, he also had some features of small bowel obstruction. The combination of these made a preoperative diagnosis difficult.

Diagnosis of sclerosing mesenteritis may be complex and involves appropriate clinical and radiographic analysis. However, for a definitive diagnosis, biopsy and histological confirmation may be necessary. The main histological features are fat necrosis, chronic inflammation and fibrosis, which may all occur together, but there is a predominance of one of these features, as shown in Table 3. The histological diagnosis, as in our case, can be improved with immunohistochemistry techniques using smooth muscle actin, cytokeratin and vimentin[4,9].

Sclerosing mesenteritis has been best diagnosed radiologically with multidetector CT and magnetic resonance imaging (MRI). The two main CT features are the "fat-ring" sign (area of fat around the mesenteric vessels)[8,14-16] and the presence of a tumoral pseudocapsule[1,2,7,8]. The other CT findings include changes of increased attenuation in the mesentery, low-attention foci representing fat necrosis and fibrosis, and development of a solid mass encasing the mesenteric vessel calcification in the center of the necrotic portion of the mass[8,14,16]. The role of CT angiography is also helpful in delineating the relationship of the mass to the mesenteric vasculature. Despite our patient having a CT scan, there were no typical radiological features of this condition noted.

Ghanem *et al*[17] have recently described the role of MRI in finding a fibrous capsule in retractile mesenteritis. The role of fluorine-18 fluorodexoyglucose positron emission tomography has also been reported in the literature as a new and upcoming image modality in the detection of sclerosing mesenteritis[15].

Management of sclerosing mesenteritis is dependent on the stage and hence histological findings of the disease (Table 3). In the early stages, when fat necrosis is the main feature, it tends to settle spontaneously without treatment. As the disease progresses and chronic inflammation predominates with undeveloped fibrosis, various agents have been used alone or in combination. The role of early treatment with cyclophosphamide has been reported in two cases with promising results, without signs of recurrence[18]. A single case study has shown the positive effect of the combination of corticosteroids and azathioprine[19]. Cuff *et al*[3] and Genereau *et al*[20] have demonstrated the effective management of colchicines, together with corticosteroid, in a series of symptomatic patients. In the final stages, when fibrosis overshadows the fat necrosis and chronic inflammation, surgical intervention, which includes partial resection, bypass, and colostomy may become necessary. Overall, surgical treatment should be limited to establishing a diagnosis or treatment of complications[3,7]. In our patient, the complications of small bowel obstruction and a localized perforation occurred, which required resection. It is worthy of note that the intraoperative findings can mimic a malignant process as highlighted in the report by Mathew *et al*[2]. Despite the fact that our patient succumbed to his postoperative complications, the overall prognosis published in the literature is favorable for this condition.

Our case illustrates that the diagnosis of sclerosing mesenteritis can be difficult to make preoperatively, especially when other procedures, such as colonoscopy, have been carried out recently, which by itself has the risk of causing iatrogenic perforation. The presentation and management of this condition can be varied and this depends upon the underlying stage of the histological process.

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Bile duct ligation in rats: A reliable model of hepatorenal syndrome?

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Abstract

The two most widely used experimental models of advanced liver disease are the administration of carbon tetrachloride, and common bile duct ligation (BDL), however, neither has been systematically evaluated as a model of hepatorenal syndrome (HRS). The BDL model in rats, studied at diverse time points, induced a progressive renal dysfunction without structural changes in the kidney. The authors concluded that BDL is a good model for further studies of HRS and its treatment. However, the renal impairment observed at the acute phase of the BDL model is based on a different pathophysiology than that of HRS. Specifically, in acute obstructive jaundice, cholemia predominates over parenchymal liver disease (reversible at this stage without portal hypertension or cirrhosis) and independently induces negative inotropic and chronotropic effects on the heart, impaired sympathetic vasoconstriction response and profound natriuresis and diuresis that might lead to volume depletion. In addition, systemic endotoxemia contributes to the prerenal etiology of renal impairment and promotes direct nephrotoxicity and acute tubular necrosis. On the other hand, the renal failure observed in the chronic BDL model (with development of biliary cirrhosis, portal hypertension and ascites) shares pathophysiological similarities with HRS, but the accordance of the chronic BDL model to the diagnostic criteria of HRS (e.g. absence of spontaneous bacterial peritonitis, no renal function improvement after plasma volume expansion) should have been confirmed. In conclusion, we think that the BDL model is not suitable for the study of the natural history of HRS, but the chronic BDL model might be valid for the study of established HRS and its potential therapies.

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Key words: Obstructive jaundice; Rats; Bile duct ligation; Hepatorenal syndrome; Renal failure

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TO THE EDITOR

We read with great interest the article recently published in *World J Gastroenterol* by Dr. Pereira *et al*[1], which evaluated the reliability of the bile duct ligation (BDL) model in rats for the study of hepatorenal syndrome (HRS). The authors found that this experimental model induces progressive renal dysfunction without structural changes in the kidney, and they suggested that BDL in rats emerges as a good model for further studies of HRS pathophysiology and treatment. Upon reading this very interesting study, a number of questions a rose as to whether the renal dysfunction observed after BDL really represents HRS.

HRS is defined as a specific type of functional renal failure complicating advanced liver disease (e.g. decompensated cirrhosis, alcoholic hepatitis, or acute liver failure)[2,3]. The pathogenesis of this syndrome is the result of an extreme underfilling of the arterial circulation secondary to an arterial vasodilation located in the splachnic circulation. This underfilling triggers a compensatory response with activation of vasoconstrictor systems leading to intense renal vasoconstriction, especially to the renal cortex, and finally the glomerular filtration rate is decreased in the absence of underlying kidney pathology whilst tubular function is preserved[4]. Consequently, this specific type of functional renal failure observed in advanced liver disease must be differentiated by a number of non-functional causes of renal failure in this setting, e.g., other causes of prerenal azotemia or acute tubular

necrosis[3,4]. The distinction between HRS and other causes of renal failure that may occur in cirrhosis is problematic mainly due to the lack of a specific diagnostic test. A number of commonly used urinary indices (e.g. urinary sodium or osmolarity) cannot reliably distinguish HRS and acute tubular necrosis in the setting of cirrhosis, making this differentiation especially difficult[3,4]. For these reasons, the diagnosis of HRS is currently based on the exclusion of other disorders that could lead to renal failure in cirrhosis including shock (septic or hypovolemic), ongoing bacterial infection, fluid losses and current treatment with nephrotoxic drugs[4]. An additional major criterion that should be fulfilled is that no sustained improvement in renal function occurs after expansion of plasma volume. In their study, the authors have not examined the accordance of the BDL model with these criteria, instead they characterize the BDL-induced renal dysfunction as "HRS" based solely on the absence of histopathological changes in the kidneys and on evaluation of diverse urinary indices. The histological analyses performed in the kidney are not described in detail but it is generally stated that BDL rats exhibited normal renal histology under the light microscope. This finding contradicts most previously published reports, which describe significant histological alterations, predominantly located in tubular epithelial cells, at various intervals of obstructive jaundice[5-10]. Furthermore, as already stated, urinary indices represent only minor and dispensable criteria for the diagnosis of HRS[4]. Taking into consideration these issues, a number of uncertainties are raised regarding the reliability of the BDL model for the study of HRS, which become more evident when considering the pathophysiology of renal failure complicating obstructive jaundice.

In rats, double ligation of the common bile duct with its dissection between the ligatures produces a well established experimental model of: (1) acute obstructive jaundice, studying different time points up to 2 wk after BDL; (2) progression of biliary fibrosis to cirrhosis, studying diverse time intervals up to 4 or 6 wk after BDL; and (3) secondary biliary cirrhosis, at 4 or 6 wk after BDL[11,12]. BDL produces a combined model of cholemia and parenchymal liver disease. The magnitude of contribution of any one factor in remote organ injury and systemic complications depends on the duration of the biliary obstruction. In acute obstructive jaundice, the liver presents typical changes of obstructive cholangiopathy, in the absence of cirrhosis or portal hypertension, which are at least partly reversible if biliary drainage is performed at this stage[13,14]. This initial phase of surgical jaundice is characterized by the effects of severe cholestasis and cholemia due to total obstruction of bile flow, with intestinal barrier failure and decreased reticuloendothelial system function. This predisposes the test subject to portal and systemic endotoxemia and susceptibility to postoperative septic complications and renal dysfunction[15,16]. Severe cholemia, predominantly and independently of liver parenchymal disease, affects the integrity of the cardiovascular system by inducing;s (1) negative inotropic and chronotropic effects on the heart[17,18]; (2) impaired sympathetic vasoconstriction response[19,20]; and (3) profound natriuresis and diuresis that may lead to volume depletion[21,22]. These factors produce systemic hypotension and renal dysfunction, especially when an interventional approach for the release of biliary obstruction is performed[22]. The etiology of renal impairment in this setting is profoundly prerenal and occurs in the absence of advanced and irreversible liver disease. In addition, it has been shown repeatedly that acute obstructive jaundice is universally complicated by extended bacterial translocation with portal and systemic endotoxemia[23-25]. These phenomena activate a systemic inflammatory response characterized by the release of numerous cytokines and proinflammatory mediators, which may lead to the development of the septic syndrome and multiple organ damage[26]. Endotoxin-induced renal injury is not only functional, through induction of a hypotensive response, but endotoxin also exerts direct nephrotoxic effects. The result is renal injury clearly characterized by histological alterations of acute tubular necrosis[27,28]. Given the central role of endotoxemia in obstructive-jaundice-induced systemic complications[16,25], we would expect that renal failure in obstructive jaundice would be accompanied by tubular injury. Despite the authors' findings of normal renal histology in BDL rats, there are numerous previous studies showing that the acute BDL model induces renal histopathological changes, predominantly in the tubular epithelium (acute tubular necrosis)[5-10]. For all these reasons, we think that the pathogenesis and the type of renal dysfunction that is observed during the acute phase of BDL are different from what we mean by the term HRS.

With regard to the chronic phase of BDL (after 4 wk of biliary obstruction), apart from cholemia, the factor severe parenchymal liver disease comes into play, significantly contributing to renal dysfunction[22]. This phase is characterized by development of biliary cirrhosis with portal hypertension, and ascites and more closely resembles clinical conditions complicated by HRS[11]. However, cholemia still exists to a considerable extent and acts on renal hemodynamics. Endotoxemia, with its consecutive systemic inflammatory response and bacterial translocation to remote organs, also exists, interacting with advanced liver disease per se in the development of renal dysfunction. Is the final result on kidney the development of HRS? In order to give a reliable answer to this question we should examine the accordance of the BDL model with the well-established diagnostic criteria of HRS, as we would do in a clinical setting. Rats with BDL for more than 4 wk are cirrhotic with ascites and have increased rates of bacterial translocation; therefore, spontaneous bacterial peritonitis should be excluded as an underlying cause of renal failure. We should also demonstrate the lack of response of renal function to volume repletion by isotonic saline. If these prerequisites are fulfilled, we would be more certain to characterize the renal failure observed in the chronic phase of BDL as HRS.

In conclusion, we think that the BDL model is not

suitable for the study of the natural history of HRS, because at the acute phase of extrahepatic cholestasis, the pathophysiology of the observed renal impairment seems to be different from that of HRS. However, renal failure observed in the chronic BDL model shares pathophysiological similarities with HRS. The accordance of the chronic BDL model to the diagnostic criteria of HRS, if confirmed, may provide us with a valuable experimental model for the study of established HRS and its potential therapies. Another important issue that remains to be elucidated is the comparison of the BDL model with the carbon tetrachloride model of cirrhosis, in order to examine the potential superiority of one model over the other for the study of HRS.

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Events Calendar 2008-2009

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January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
6th International Meeting
Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

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Advanced Courses - European Institute of Telesurgery EITS - 2008
Strasbourg, France
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S
April 3-5, November 27-29
Laparoscopic Digestive Surgery

June 27-28, November 7-8
Laparoscopic Colorectal Surgery

July 3-5
Interventional GI Endoscopy Techniques
Contact address for all courses:
E-mail: info@eits.fr

International Gastroenterological Congresses 2009
March 23-26, Glasgow, Scotland
Meeting of the British Society of Gastroenterology (BSG)
E-mail: bsg@mailbox.ulcc.ac.uk

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

November 21-25, London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

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For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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Instructions to authors

GENERAL INFORMATION

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Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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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

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462

PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

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

Patent (list all authors)

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

Statistical data

Write 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 υ (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

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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EDITORIAL

New technologies in gastrointestinal research

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Abstract

This issue presents different new techniques aiming to increase our understanding of the gastrointestinal system and to improve treatment. The technologies cover selected methods to evoke and assess gut pain, new methods for imaging and physiological measurements, histochemistry, pharmacological modelling *etc*. There is no doubt that the methods will revolutionize the diagnostic approach in near future.

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Key words: Pain; Gut; Brain; Sphincter; Imaging; Drug

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New technologies are emerging in gastroenterology. During the last decade, methods such as intraluminal ultrasound, other imaging modalities and high resolution manometry have become available for routine work, dramatically improving the ability to correctly diagnose and treat gastroenterological diseases. Experimental methods to assess the sphincter regions and the pain system have also become available with the possibility to get more insights into basic physiological mechanisms and understanding of how drugs affect the gastrointestinal tract. This has lead to an improvement in the treatment of patients with both organic and functional diseases. Unfortunately, many of these methods are still only available in the most advanced laboratories, but commercial systems are currently being developed with the aim of getting a more widespread use of the most promising techniques.

In the current issue of *World J Gastroenterol*, different techniques which are either commercially available or in development are presented. The technologies cover the selected methods to evoke and assess gut pain experimentally and in the clinic[1]; neuroimaging of the brain-gut axis[2]; new methods for ultrasound and imaging[3]; devices to assess sphincter functions and force measurements[4-6]; emerging experimental methods to measure blood flow and histochemical changes in the tissue[7,8]; manometry and impedance measurements as well as pharmacological and modelling techniques[9,10]. There is no doubt that these methods will revolutionize the diagnostic approach in near future. In particular, combining the different techniques with development of miniature equipment will lead to multimodal experimental procedures that can be done in one procedure with less discomfort and complications for the patients. Today, it is theoretically possible to combine probes for manometry, imaging, assessment of sphincter function and axial force in the oesophagus with endoscopy (including biopsies) and comprehensive testing of the pain system (including electrophysiological assessment of the brain-gut axis), all in one procedure. In the current issue, examples of such combined techniques are demonstrated. It has been shown that multimodal assessment may lead us to much a better understanding of diseases and symptoms in various patient groups. Individual genetic and environmental factors among others have now led to the understanding that patients with the same disease do not necessary respond to standard treatments. New pharmacological methods giving insight into drug mechanisms in individual patients are available and the methods provide the possibility for tailoring specific and individualized treatment in the near future. Naturally, there is still a long way to go before the ideal probes and equipment are available for general use; but with the necessary knowledge exchange and open-mindedness in the research community, there is no doubt that near future will revolutionize the way we look at the gastrointestinal tract and treat our patients.

The papers in the current issue show how innovative development and research can bring new ideas as well as refinement of older techniques into play in the gastrointestinal tract. The clinician should have at least some knowledge of these techniques as a close relationship and sharing of ideas between research and

the clinic are a sine qua none to find the right indications for the methods. In the research community, there are many more emerging new methods than those dealt with in this issue; but with the main focus on the upper gastrointestinal tract, the selected techniques represent broadly the evolution and forthcoming procedures in this important field.

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New technologies in the gastrointestinal clinic and research: Impedance and high-resolution manometry

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Abstract

The last five years have been an exciting time in the study of esophageal motor disorders due to the recent advances in esophageal function testing. New technologies have emerged, such as intraluminal impedance, while conventional techniques, such as manometry, have enjoyed many improvements due to advances in transducer technology, computerization and graphic data presentation. While these techniques provide more detailed information regarding esophageal function, our understanding of whether they can improve our ability to diagnose and treat patients more effectively is evolving. These techniques are also excellent research tools and they have added substantially to our understanding of esophageal motor function in dysphagia. This review describes the potential benefits that these new technologies may have over conventional techniques for the evaluation of dysphagia.

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Key words: Dysphagia; Multichannel intraluminal Impedance; Bolus transit; High-resolution manometry; Esophagogastric junction; Achalasia

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INTRODUCTION

The evaluation of a patient with dysphagia should begin with a careful evaluation for structural causes for obstruction, such as webs, rings, strictures and mass lesions. Once these entities are ruled out with either endoscopy or fluoroscopy, the work-up should shift its focus to defining esophageal motor function and bolus transit abnormalities. Although fluoroscopy can provide information on bolus transit, it is burdened by the requirement of radiation, and the inability to detect the pressure profile of the peristaltic event. Currently, only manometry can define the pressure profile of both peristalsis and LES relaxation. However, this technology is limited by an inability to provide information on bolus transit and emptying. Furthermore, conventional manometry has been hindered by a lack of standardization of methodology and conflicting notions of how to analyze the data. Direct evidence of this predicament can be found in a recent publication highlighting the poor inter-observer agreement in the analysis of clinical manometry; even amongst expert practitioners[1].

Given the above observations, two technologies have been developed that have the potential to improve the management of dysphagia: high-resolution manometry and intraluminal impedance monitoring. While high-resolution manometry is an evolution of current manometric techniques, impedance monitoring is a new technology that complements manometric data by providing information on bolus transit without the need for radiation or an additional test. These two techniques have advanced our understanding of esophageal motility and it is very likely that they will also improve clinical management.

MULTICHANNEL INTRALUMINAL IMPEDANCE (MII)

MII: Technical aspects

As mentioned previously, fluoroscopic evaluation

of the esophagus is an excellent tool to assess the intraluminal anatomy of the esophagus as well as bolus transit. However, it requires radiation and also lacks the detail to define and classify esophageal motor function. Intraluminal impedance monitoring was devised to circumvent the requirement of radiation in assessing bolus transit by monitoring intraluminal resistance. Impedance monitoring works by using an alternating current generator to apply an electrical potential between pairs of metal electrode rings separated by an isolator. The electrical current loop can only be closed through the conduction of electrical charges by the surrounding material bridging the two metal electrode rings. Air, liquid (saline/refluxate), and the esophageal mucosa each have unique impedance characteristics, thereby allowing definition of which material resides between each pair of electrodes. Air is highly resistant to current flow and, thus, has a high impedance value, while saline and gastric juice have relatively low resistance to flow and, thus, have a low impedance value. Esophageal mucosa has an intermediate impedance range and, thus, serves as a baseline during monitoring.

By dispersing the impedance electrodes along a catheter, and defining impedance changes at adjacent pairs of rings, one can determine the direction of bolus transit within the esophagus and also document whether complete bolus clearance has occurred (Figure 1)[2-4]. Studies using combined fluoroscopy and impedance have validated the convention that liquid bolus entry is characterized by a 50% drop in impedance at the recording site while bolus exit is characterized by a return to at least 50% of baseline[5,6]. Studies assessing the correlation between simultaneous barium videoesophagram and impedance revealed agreement in over 97% of swallows for determining normal bolus transit or retrograde escape and stasis[7]. Thus, this technique provides qualitative evidence of esophageal emptying. However, it does not provide quantitative data regarding volume.

MII: Investigations into dysphagia pathogenesis

An important first observation using combined MII and conventional manometry was focused on describing bolus transit patterns associated with various esophageal motor patterns. Tutuian *et al*[8] described an experience in 350 patients presenting for evaluation of esophageal function. Bolus transit was assessed based on conventional manometric criteria of swallowing function. All patients with manometrically defined achalasia and scleroderma had abnormal bolus transit. In contrast, only half of the patients with ineffective esophageal motility (IEM) and spasm had abnormal bolus transit. The majority of patients with intact peristalsis and various LES abnormalities had normal bolus transit. The authors theorized that impedance monitoring could potentially categorize esophageal motor abnormalities into more clinically relevant groups based on abnormalities of both bolus transit and pressure as opposed to pressure alone.

Looking to provide more focused information regarding a clinical role for impedance, Tutuian *et al*[9] analyzed 71 subjects with DES and characterized them based on both their motor function and ability to obtain complete bolus transit[9]. Their findings suggested that DES patients with dysphagia were more likely to have abnormal bolus transit than DES patients who presented with predominant chest pain. Furthermore, they also noted that DES patients presenting with chest pain were not only more likely to have normal bolus transit, but also exhibited higher contraction amplitudes. These observations are intriguing as it appears that impedance may help define treatment strategies for various patient groups: chest pain patients with extremely high contractile amplitudes may be a sub-population amenable to treatments with nitrates and calcium channel blockers; while patients with impaired bolus transit may require alternative therapies.

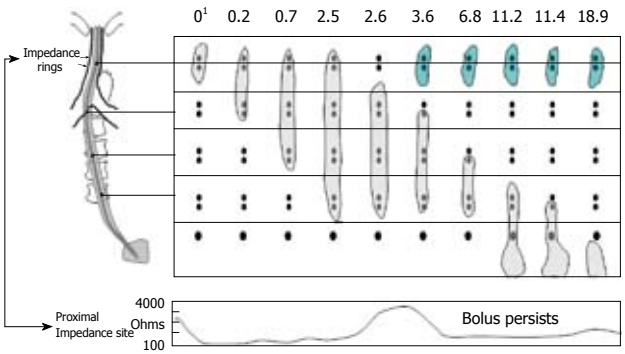

Figure 1 Example of retrograde escape and proximal stasis on simultaneous multichannel intraluminal impedance and fluoroscopy. The fluoroscopic bolus is represented by the gray bolus at each time interval during the swallow and the green bolus during retrograde escape. The bottom panel is a single impedance tracing at the proximal location. The bolus is present at the proximal impedance site until 2.6 s where the bolus tails moves distal to the two rings at that level and the impedance tracing returns to baseline. At 3.6 s, the bolus (green) reenters the recording site and once again the impedance drops consistent with bolus retention. Modified from Imam *et al*[7]. [1]Time in seconds.

MII: Clinical aspects

The clinical protocol for combined impedance/manometry is similar to the standard conventional manometric protocol with the exception that normal saline is used for the 10 liquid swallows and there is an additional portion of the study that utilizes viscous swallows. Saline is used because its high conductivity provides a contrast in impedance between the liquid bolus and the esophageal wall, while the viscous bolus is typically a gel provided by the manufacturer or another food, such as yogurt or apple sauce. Impedance parameters calculated for the evaluation of bolus transit using liquid and viscous swallows are: (1) total bolus transit time (between bolus entry at 20 cm above the LES and bolus exit at 5 cm above the LES); (2) bolus presence time (the interval between bolus entry and bolus exit at each impedance-measuring site); and (3) segmental transit times (the interval between bolus entry at a given level above the LES and bolus exit at the next most distal level)[10]. Swallows are classified as having: (1) complete bolus transit if bolus exit is recorded in all three distal impedance-measuring sites or (2) incomplete bolus transit if bolus exit is not

identified at one or more of the three distal impedance-measuring sites.

Normal values for both liquid and viscous swallows have been reported by different groups using slightly different techniques. Tutuian *et al*[10]. performed combined impedance/manometry on 43 normal healthy subjects and analyzed 10 liquid and 10 viscous swallows to determine normative ranges for total bolus transit time and the percent of swallows associated with complete bolus transit. Their results revealed that total bolus transit time for both liquid and viscous swallows were 12.5 s and greater than 90% of individuals had > 80% of the liquid swallows associated with complete bolus transit and > 70% of the viscous swallows associated with complete bolus transit[10]. Two other studies in normal controls revealed similar bolus transit parameters and thus, it appears that this technology is reproducible in normal subjects[11,12].

MII: Future directions

Although available data suggests that abnormal bolus transit occurs in many patients presenting with dysphagia, there is no clear data supporting an association of abnormal bolus transit, and the perception of dysphagia. Thus, future research should focus on the mechanisms by which abnormal bolus transit elicits symptoms. Such studies should consider the role of sensitivity, anxiety state and other potential confounders that may modulate symptoms. In addition, studies focused on whether or not abnormal bolus transit can predict clinical outcomes in dysphagia patients are also needed to support the role of impedance in clinical practice.

New technologies incorporating impedance techniques are also evolving that can provide more detail regarding anatomy and mechanical properties of the esophageal body[13,14]. The functional lumen imaging probe (FLIP) was created by McMahon *et al*[13,14] to measure cross-sectional area of the esophagus at extremely small intervals. This technique applies computer software that converts the high-resolution planimetry data into a computer animation that recreates the geometry of the anatomic zone being studied. In addition, a pressure/distention relationship can be measured to define elasticity and compliance of the lumen wall and, thus, represents the first dynamic technique to profile both anatomy and function of the esophagus with a single device. Although these devices are currently only available for research purposes, it is likely that they will be incorporated into clinical devices along with high-resolution impedance and topographic display methods.

HIGH-RESOLUTION MANOMETRY

Esophageal manometry is considered the "gold standard" for assessing esophageal motor function[15]. Accordingly, the current diagnostic classification of esophageal motor disorders is based almost entirely on manometric patterns of abnormal peristalsis and LES function[16]. Conventional manometry typically utilizes 3-8 pressure sensors positioned within the esophageal lumen to assess the contractile pattern during water swallows. A variety of sensor technologies exist, including solid-state transducers, circumferentially sensitive transducers, perfused ports, and the Dentsleeve device. The heterogeneity of the sensor types and lack of consensus regarding the optimal spacing of sensors may be partially responsible for the poor intra- and inter-observer reproducibility reported in the literature[1]. Thus, refinements in manometry are needed to improve reproducibility and accuracy of this "gold standard".

HRM: Technical aspects

High-resolution manometry is not a new technology and represents a modification of existing technology to provide greater detail by utilizing a vastly increased number of sensors spaced closely together. Thus, not only time, but also the spatial domain of the pressure profile within the esophagus can be captured as a continuum after interpolation between the sensors. The ideal system for esophageal studies should span from the pharynx to the stomach with sensor separation of no more than a centimeter apart within and around the sphincters and a temporal frequency response matched to the zone of the esophagus in which the sensors reside. High-resolution manometry can be adapted to work with any transducer technology, as long as the recording fidelity of the sensor is adequate. The frequency response required to reproduce esophageal pressure waves with 98% accuracy is 0-4 Hz, while that required for reproducing pharyngeal pressure waves is 0-56 Hz[17]. Expressed in terms of maximal recordable $\Delta P/\Delta t$, 300 mmHg/s is sufficient for studying the esophageal body while the pharynx will require a $\Delta P/\Delta t$ of 4000 mmHg/s for the pharynx.

Early studies incorporating high-resolution manometry utilized water perfused systems due to availability of appropriate multilumen extrusions, the flexibility of the sensor spacing, and the cost of solid-state pressure sensors. However, the response characteristics of the water perfused sensors were technically limited for studying the pharynx or for measuring detailed pressure gradients through both the upper and lower esophageal sphincters. Thus, the ideal system would incorporate solid-state sensors, which have become clinically available. The advantages of this type of high-resolution system are: (1) a simplified procedural set up with improved sphincter localization; (2) elimination of movement artifact; (3) simplified data interpretation; and (4) ability to perform more sophisticated analysis of esophageal function. These attributes alone make this technology more user-friendly and efficient. Thus, it has the potential to replace conventional manometry utilizing a line tracing format.

The vastly increased quantity of data and the confusing presentation of multiple overlapping tracings spaced closely together presents new challenges for interpreting high-resolution manometry. Thus, new algorithms have been devised to provide a seamless dynamic representation of pressure at every axial position from the hypopharynx through the EGJ. Advances in computerization and graphic data presentation have been adapted so that esophageal contractile activity following a swallow can be portrayed

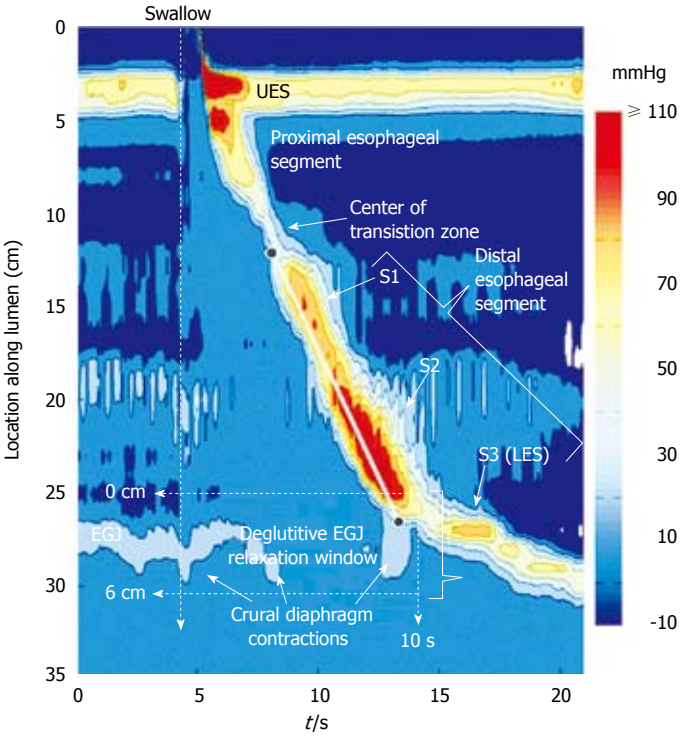

Figure 2 Typical swallow pressure topography spanning from the pharynx (locations 0-2 cm) to stomach (locations 29-35 cm) of a normal subject with normal peristalsis and normal EGJ relaxation. The transition zone, demarcating the end of the proximal esophageal segment (striated muscle) and the beginning of the distal esophageal segment (smooth muscle), is readily identified as a pressure minimum. Note that the distal segment, in fact, has three sub-segments (S1, S2, S3) within it, each with an identifiable pressure peak. Sub-segment 3, the LES, contracts at the termination of peristalsis and then descends back to the level of the CD as the period of swallow-related esophageal shortening ends. The onset of the deglutitive relaxation window is at the onset of upper sphincter relaxation while the offset is 10 s later. The spatial domain within which EGJ relaxation is assessed (the eSleeve™ range) is user defined, spanning at least 6 cm, depending on the extent of esophageal shortening after the swallow. The contractile front velocity (CFV) is the slope of the line connecting the black circle-points on the 30 mmHg isobaric contour at the proximal margin of S1 and the distal margin of S2.

in the format of color isobaric contour topographic plots (Figure 2). Clouse and Staino were the first to apply this technique in the esophagus and developed a methodology to interpolate and convert this information into topographic plots[18]. This data presentation format is more akin to an imaging technique, as opposed to conventional manometric tracings, and has the potential to greatly simplify and standardize the clinical manometric study.

HRM: Investigations into dysphagia pathogenesis

Topographic analysis of HRM data has clearly advanced our knowledge of esophageal motor function. The recent application of topographic analysis to esophageal peristalsis demonstrated that progression through the esophageal body is not seamless. Rather, it is comprised of a sequence of contractile events occurring in four discrete pressure segments (Figure 2). The first segment represents the striated muscle component of the proximal esophagus, and extends from the UES to the first pressure trough in the region of the aortic arch. This trough, representing the transition zone is usually easily identified. The distal portion of the esophagus is considered the smooth muscle dominant portion, and can be separated into two overlapping neuromuscular segments. The fourth contractile segment encompasses the LES. This segmental configuration was not appreciated by conventional manometry and underscores the strength of topographic analysis of manometric data[18-20].

Within the esophageal body one of the early achievements of HRM was the understanding of the transition zone in the mid esophagus, not just as the locus of the nadir in peristaltic pressure amplitude, but also as a physiological transition between propagated contractions of completely distinct physiology[21]. The proximal segment is that dominated by striated muscle, while the distal segment is smooth muscle. The proximal contraction is attributable to sequenced activation of motor neurons in the medulla, while the distal contraction in the smooth muscle is sequenced as a function of the balance between the excitatory and inhibitory interneurons of the myenteric plexus. This enhanced understanding of the transition zone can also account for the distinct pathology in which there is an abnormal delay between the termination of the proximal contraction and the origination of the distal contraction or a spatial gap between the two, as an explanation for dysphagia[22].

One of the first important investigations that took advantage of the detail HRM provides in defining simultaneous pressure across anatomic zones, was performed by Pal *et al.* to define pathologic constriction across the UES[23]. Their findings suggested that intrabolus pressure was an important marker of impaired bolus transit, and represented constriction at the UES. Our group built upon this work, utilizing combined HRM and fluoroscopy at the upper esophageal sphincter to determine if manometry alone could be leveraged to accurately predict bolus transit across the EGJ in patients with a spectrum of pathological conditions[24]. Using the normative data for esophago-gastric flow permissive time in 20 controls and mathematical modeling data of antegrade esophageal emptying[25], we performed a ROC analysis for the predictive value of flow permissive time that is optimal to predict complete clearance. Our findings suggest that a cut-off value of $\leqslant$ 2.5 s had the best predictive value for incomplete clearance with a sensitivity of 86% and specificity of 92%. We speculate that intrabolus pressure elevations above the EGJ may also be useful in defining pathologic constriction or impaired EGJ opening and future work should be focused on defining normative values for intrabolus pressure.

HRM: Clinical aspects

Given the fact that high-resolution manometry is a refinement of an already existing clinical tool, it can be

Table 1 Classification of individual swallows based on pressure topography criteria

Classification	Criteria
EGJ Deglutitive Relaxation (referenced to gastric pressure)	
Normal relaxation	4 s Integrated Relaxation Pressure (IRP) < 15 mmHg
Impaired relaxation	4 s IRP ⩾ 15 mmHg
Distal Segment Contraction (referenced to gastric pressure)	
Normal	⩽ 2 cm defect in the 30 mmHg isobaric contour, Contractile Front Velocity (CFV) < 8 cm/s, Intrabolus Pressure (IBP) < 15 mmHg, and Distal Contractile Integral (DCI) < 5000 mmHg × s × cm
Mild peristaltic defect	Normal appearing wavefront propagation with a 2-5 cm defect in the 30 mmHg isobaric contour
Severe peristaltic defect	Evidence of wavefront propagation with a ⩾ 5 cm defect in the 30 mmHg isobaric contour
Absent peristalsis	No propagating contractile wavefront and minimal (< 3 cm) contractile activity or pressurization greater than the 30 mmHg IBC
Nutcracker	DCI > 5000 & < 8000 mmHg × s × cm
Spastic nutcracker	DCI > 8000 mmHg × s × cm
Spasm	Simultaneous contraction (CFV > 7.5 cm/s)
Elevated intrabolus pressure	IBP > 15 mmHg compartmentalized between the EGJ and the peristaltic wavefront
Panesophageal pressurization	Esophageal pressurization UES to EGJ with > 30 mmHg IBP

easily substituted for conventional manometry in our standard evaluation of esophageal motor disorders. The clinical protocol for high-resolution manometry is almost identical to standard conventional manometry with the obvious exclusion of the initial pull-through protocol to localize the LES. This portion of the initial intubation procedure and positioning of the catheter is not required due to the increased number and close proximity of the sensors making the sphincters easily identifiable on the topographic pressure plots.

With the adoption of HRM technology and pressure topography display methodology, the classification of esophageal motility that was developed for conventional manometric systems needs to be reconsidered. Although conventional metrics can be easily measured using simple software programs to convert the topographic data back to conventional line tracings and then applying a conventional analysis to a selected set of the line tracings, this method ignores the incremental gain in information obtained from the patterns presented by the pressure topographic plots. The alternative approach is to build an analysis and classification scheme that parallels conventional manometric classification, but also enhances it based on the strengths of the technology. To that end, we recently completed a comprehensive characterization of esophageal HRM data using novel analysis paradigms devised for pressure topography interpretation. We analyzed 75 normal subjects to develop normative ranges and applied this analysis system to 400 patients[26]. The major conclusions from that work, along with relevant contributions from other research groups, has allowed the initial formulation of an analysis and classification system for clinical practice and will be summarized in the following sections and tables.

The approach to analyzing and classifying HRM studies parallels conventional manometric technique in that it is focused on defining sphincteric and esophageal body function. However, new analysis paradigms have been created to assess deglutitive EGJ relaxation and peristaltic integrity and vigor with higher accuracy and detail. Basal EGJ relaxation pressure is measured using similar methodology to that used in conventional manometry; by assessing the mean end-expiratory pressure during a sufficient baseline period at the beginning of the study. Defining EGJ relaxation, however, has been modified to quantify EGJ relaxation pressure during the entire deglutitive period. Although a single nadir pressure measurement can be easily measured, bolus emptying and flow through the EGJ is not instantaneous and may take up to 4 to 5 s based on the volume swallowed[24]. Thus, deglutitive relaxation pressure is now quantified by measuring the 4 s integrated relaxation pressure (IRP), which represents the lowest 4 s cumulative pressure values for the deglutitive time period through the anatomic zone defining the EGJ. Normative values for this parameter were derived form 75 normal controls (Table 1) and this measurement was shown to be superior to a single nadir measurement and the continuous 3 s nadir pressure[27].

Recognizing that esophageal bolus transport is effected by the interaction of resistance through the EGJ, intrabolus pressure, and esophageal closure pressure behind the bolus[28], the second step of the analysis focuses on defining peristaltic integrity. Given previous data suggesting that pressures greater than 30 mmHg are almost uniformly associated with complete bolus transit[29,30], this threshold value was applied to define an intact peristaltic wavefront. This analysis is facilitated by the generation of 30 mmHg isobaric contour plots that delineate a pressure domain such that all pressures above this threshold value are circumscribed by a dark line. Normal swallows should have a seamless intact 30 mmHg isobaric contour with a contractile front velocity (CFV) below 7.5 cm/s. Abnormalities of single swallows are defined by the defects in the 30 mmHg isobaric contour and the contractile front velocity (Table 1).

The vigor of the smooth muscle contraction is another component that can be quantified in detail with HRM using a parameter defined as the distal contractile integral (DCI). This parameter quantifies the contractile activity in a space-time box by multiplying the length of

Table 2 Distal esophageal motility disorders based on pressure topography criteria

Disorder	Criteria
With Normal EGJ Relaxation (mean IRP < 15 mmHg)	
Peristaltic Weakness	
Intermediate	More than 30% of swallows with mild or severe peristaltic defects, but numerically insufficient to constitute severe peristaltic weakness
Severe	⩾ 70% of swallows with severe peristaltic defects
Aperistalsis	100% swallows with absent peristalsis
Nutcracker Esophagus	Normal CFV, Mean DCI > 5000 and < 8000 mmHg × s × cm, can be localized to either distal subsegment or LES
Spastic Nutcracker	Normal CFV, Mean DCI > 8000 mmHg × s × cm
Distal Esophageal Spasm	Normal EGJ relaxation and spasm (CFV > 8 cm/s) with ⩾ 20% of swallows
Esophageal Obstruction	Increased IBP or panesophageal pressurization not associated with EGJ obstruction
With Impaired EGJ Relaxation (mean IRP ⩾ 15 mmHg)	
Achalasia	
Classic achalasia	Impaired EGJ relaxation and aperistalsis
Achalasia with esophageal compression	Impaired EGJ relaxation, aperistalsis, and panesophageal pressurization with ⩾ 20% of swallows
Spastic achalasia	Impaired EGJ relaxation, aperistalsis, and spasm with ⩾ 20% of swallows
EGJ Obstruction	
Mild	Elevated IBP (15-30 mmHg) that is compartmentalized between the peristaltic wavefront (normal, weak, or nutcracker) and EGJ
Severe	IBP > 30 mmHg that is compartmentalized between the peristaltic wavefront (normal or nutcracker) and EGJ

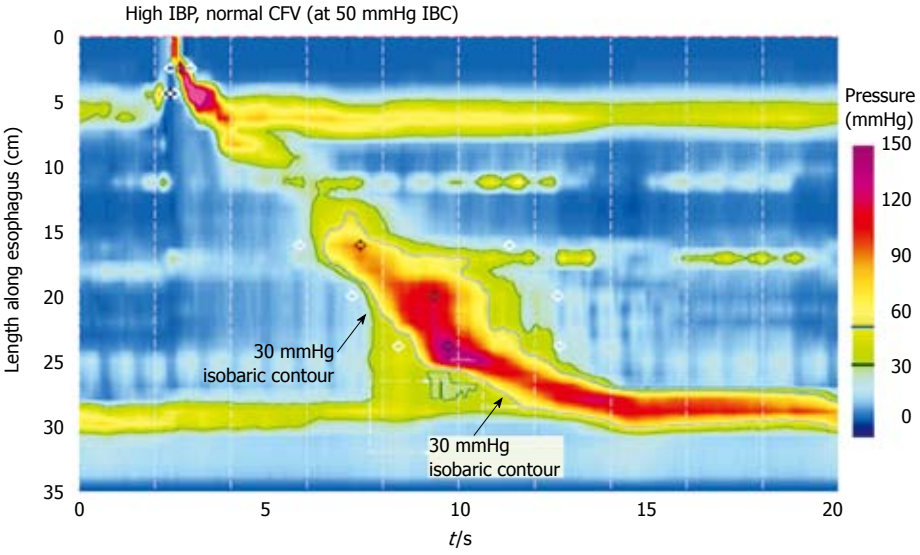

Figure 3 Defining increased intrabolus pressure using high-resolution manometry. The figure illustrates a swallow with functional obstruction at the EGJ. Note that the 30 mmHg isobaric contour line (black) deviates quickly from the 50 mmHg isobaric contour line (blue). In this case, the contractile front velocity is normal, reflecting the propagation velocity of 50 mmHg isobaric contour rather than the 30 mmHg isobaric contour. The intrabolus pressure domain is defined by the compartmentalized pressurization between the propagating contraction and the EGJ. Modified from: Pandolfino *et al*[26].

the smooth muscle esophagus by the duration of propagation of the contractile wave front, and the mean pressure value over the entire box excluding pressure signal below 20 mmHg[26]. The DCI provides much more detail than 3 isolated measurements of maximal contractile amplitude at 3-5 cm intervals, as it incorporates duration of contraction into the measurement. Although a DCI value > 5000 mmHg × s × cm exceeded the 95th percentile of normal thereby meeting the usual criterion for nutcracker esophagus, a threshold value of 8000 mmHg × s × cm distinguished a spastic nutcracker subgroup (n = 12) characterized by repetitive high amplitude contractions that was uniformly associated with dysphagia or chest pain[26]. Intrabolus pressure is another feature that can easily be defined by HRM using the isobaric contour tool to assess patterns of pressurization (Figure 3).

These measurement parameters for single swallows can be utilized to classify esophageal motor disorders[26,31]. Although HRM classification schemes have not been rigorously validated, they once again parallel conventional classification, and have the added benefit of much greater detail and an ability to characterize intrabolus pressure. Thus, it should not be difficult to incorporate these classification schemes into current clinical practice. Table 2 represents our motility laboratories version of an evolving classification scheme. Apart from changing the paradigm of categorizing peristalsis based on an intact isobaric contour, the classification of peristaltic dysfunction is very similar to conventional classification with the extra detail of distinguishing spastic nutcracker. The fundamental differences between the HRM classification system and previous conventional classification systems focuses on the sub-classification of achalasia and the category of EGJ obstruction defined by elevated IBP and impaired EGJ relaxation (Figure 3). In our analysis of 400 consecutive patients,

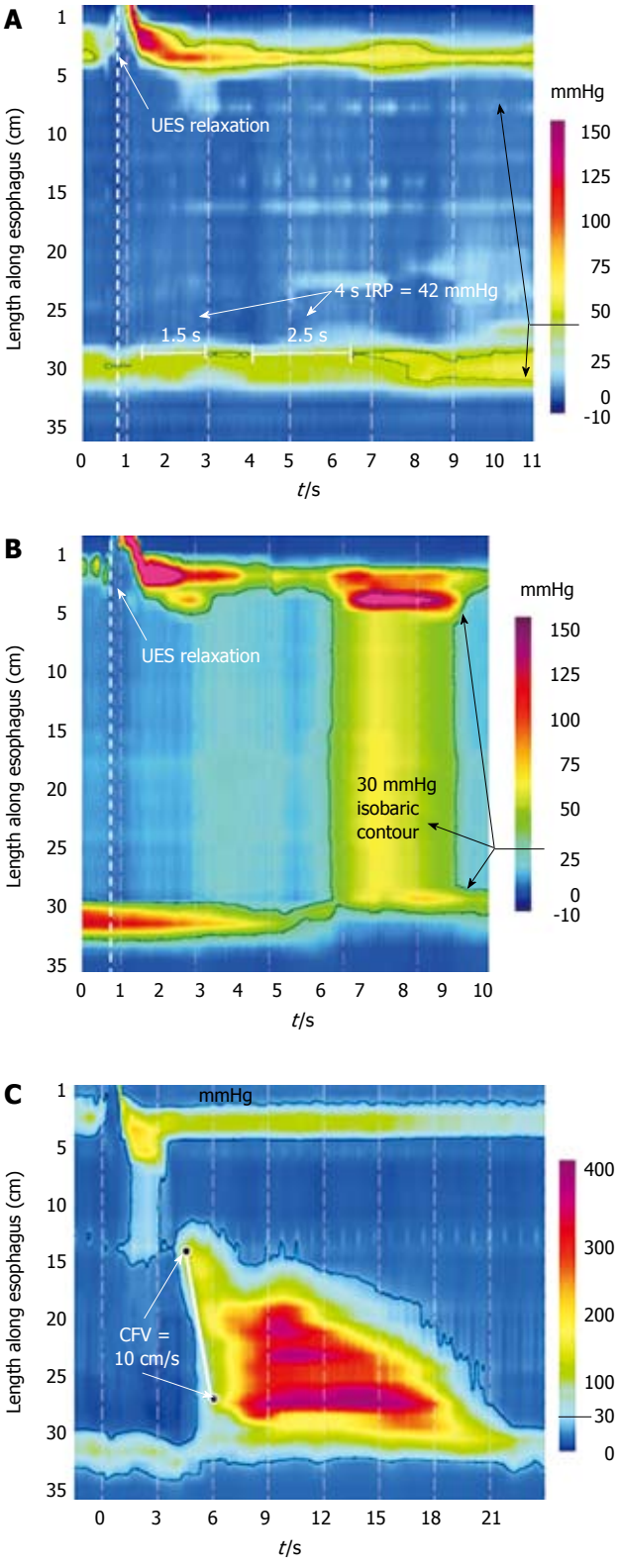

Figure 4 Achalasia subtypes based on manometric patterns of esophageal body contractility. A: Classic achalasia. There is no significant pressurization within the body of the esophagus and there is concurrent impaired EGJ relaxation (IRP of 42 mmHg in this example); B: Achalasia with compression. This subtype exhibits a rapid pan-esophageal pressurization; C: Spastic Achalasia. Although this swallow is associated with rapidly propagated pressurization, the pressurization in this case is attributable to an abnormal lumen obliterating contraction. Modified from: Pandolfino *et al*[26].

we found that 44 patients met this criteria and these patients were a heterogeneous group composed of post-fundoplication surgery, esophageal stricture, eosinophilic esophagitis and an idiopathic group that could represent an achalasia variant or pathology that could not be defined by conventional methodology[26].

A diagnosis of achalasia requires both aperistalsis and impaired deglutitive EGJ relaxation. However, there are specific subtypes that can be identified using HRM that can predict clinical outcome (Figure 4). In its most obvious form, achalasia occurs in the setting of esophageal dilatation with negligible pressurization within the esophagus (Type 1). However, despite there being no peristalsis, substantial pressurization within the esophagus can also occur. In fact, a very common pattern encountered in achalasia is of pan-esophageal pressurization (Type 2). These patients generally have a non-dilated esophagus with no obvious endoscopic or radiographic abnormalities. The other, much less common, pattern is of spastic achalasia in which there is a spastic contraction within the distal esophageal segment (Type 3). In a series of 73 consecutive achalasics, 40 (54.8%) had aperistalsis, 29 (39.7%) had pan-esophageal pressurization, and only 4 (5.5%) had spastic achalasia (Pandolfino *et al*[26]). The importance of this classification scheme was highlighted when logistic regression analysis found Type 2 to be a predictor of positive treatment response while Type 3 was predictive of negative treatment response regardless of whether treatment was medical, endoscopic or surgical.

CONCLUSION

Multichannel intraluminal impedance and HRM are new tools that can improve the accuracy and detail in describing esophageal function. These technologies should not be viewed as competing technologies, as each provides distinct information. Rather, efforts should be focused on combining these techniques, as they are largely complementary. For instance, high-resolution manometry is the best method to analyze the pressure profile of the esophagus, and certainly it should be combined with impedance monitoring if bolus transit abnormalities are shown to help define disease states and direct clinical treatment.

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Axial force measurement for esophageal function testing

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Abstract

The esophagus serves to transport food and fluid from the pharynx to the stomach. Manometry has been the "golden standard" for the diagnosis of esophageal motility diseases for many decades. Hence, esophageal function is normally evaluated by means of manometry even though it reflects the squeeze force (force in radial direction) whereas the bolus moves along the length of esophagus in a distal direction. Force measurements in the longitudinal (axial) direction provide a more direct measure of esophageal transport function. The technique used to record axial force has developed from external force transducers over in-vivo strain gauges of various sizes to electrical impedance based measurements. The amplitude and duration of the axial force has been shown to be as reliable as manometry. Normal, as well as abnormal, manometric recordings occur with normal bolus transit, which have been documented using imaging modalities such as radiography and scintigraphy. This inconsistency using manometry has also been documented by axial force recordings. This underlines the lack of information when diagnostics are based on manometry alone. Increasing the volume of a bag mounted on a probe with combined axial force and manometry recordings showed that axial force amplitude increased by 130% in contrast to an increase of 30% using manometry. Using axial force in combination with manometry provides a more complete picture of esophageal motility, and the current paper outlines the advantages of using this method.

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Key words: Axial force; Traction force; Manometry; Motility; Peristalsis; Esophageal function

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INTRODUCTION

The primary function of the esophagus is to transport ingested material to the stomach. Some of the most prevalent diseases in the esophagus relate to malfunction of transport e.g. reflux of stomach contents and motility disorders. The regulation of the normal function of the esophagus is complex, and requires fine coordination of the longitudinal muscles and circular muscles[1]. Manometry is the gold standard for the diagnosis of motility diseases in the esophagus[2]. It has been used for many decades, and provides an indirect picture of the motility patterns because it only gives information about muscle contraction or radial squeeze[3]. However, any contraction - strong or weak - will only be measured by manometry if it occludes the measuring catheter. Using data from computer models, it has been argued that shortening of the longitudinal muscle plays an important role in the mechanisms of peristalsis and that pressure amplitude per se does not give any indication of the force required to drive the bolus forward[4]. Hence manometric recordings alone are insufficient to describe and quantify esophageal motility. To improve this, and gain more knowledge, modalities such as fluoroscopy[5,6] and ultrasound[7] have been used in combination with manometry. These modalities have confirmed that parameters recorded by manometry only partly describe the peristaltic wave, but these imaging modalities do not provide quantitative information on force in either radial or axial directions[8]. Furthermore, in the clinic, these extra modalities in combination with manometry are inconvenient because multiple examinations are needed.

A more physiologically related measure that gives direct information about the motility is to record the force that pushes or propels the bolus in an axial direction towards the stomach. This method of quantifying peristalsis is referred to as propulsive force[6,9-13], traction force[6,14-17] and peristaltic force[11,18]. We refer to these concepts as axial force as this is the direction of the force in contrast to manometry, which records the radial force.

AXIAL FORCE RECORDING TECHNIQUES

The number of publications in relation to axial force is limited to less than ten, with Winship *et al*[12] being the first to publish a method that recorded the axial force of the human esophagus in 1967. They used an external force transducer connected to a plastic sphere placed in the esophagus. This enabled assessments of the esophagus' ability to propel the plastic sphere against a known resistance. Pope *et al*[11] and Schoen *et al*[18] used a mercury-in-silastic strain gauge which was placed together with a plastic sphere in the esophagus. The next development by Russell *et al*[13] was similar to previous editions though the mercury in the strain gauge was replaced by saline to reduce the effect of temperature dependence. The use of a plastic sphere did not allow a change of volume *in vivo*, hence in studies with varying bag sizes, the probe had to be redrawn, the sphere replaced and swallowed again. This could introduce some errors in terms of positioning as well as irritation and secondary contractions. Williams *et al*[14] and Pouderoux *et al*[6] and co-workers used a strain gauge, but did not describe any technical details. The next series of publications were based on the use of a miniature strain gauge, and published in the period from 1992 to 1997[6,14-17]. A new technique, based on impedance planimetry, was introduced recently (2008) by our group[19]. The principle of impedance planimetry is to create an electric field between two excitation electrodes placed in a bag with conductive fluid. Two detection electrodes placed close to each other, and midway between the excitation electrodes, measure the cross-sectional area. This is possible as the impedance between the electrodes is proportional to the distance between the detection electrodes, and inversely proportional to the conductivity of the fluid and the cross-sectional area[20]. The only variable left is the cross-sectional area, as the other parameters are constants implemented in the calibration[12]. We took advantage of our experience with impedance planimetry, and redesigned the probe to have a constant cross-sectional area, but allowed the distance between the detection electrodes to vary (Figure 1). Thus, the potential between the electrodes would be linearly related to the axial force. This design enabled easier probe construction and less technical pitfalls compared to the strain gauge design.

Axial force still needs to prove its utility in relation to high resolution manometry. It has not yet been documented how high resolution manometry and axial force correlate. We believe that despite the extra information high resolution manometry can provide, it still does not record the actual function of the esophagus and any attempt to interpret the bolus transport can be flawed. An undetected transport of the bolus can still occur with manometry becauses it mainly quantifies the circular muscles' contractions

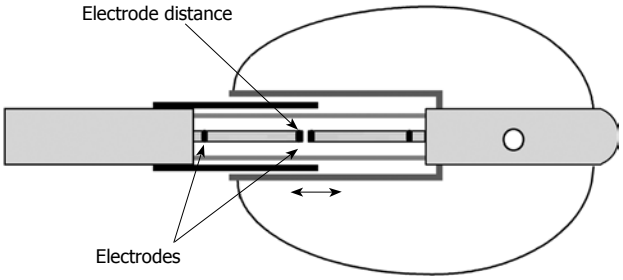

Figure 1 The redesign of impedance planimetry has enabled measurements of axial force instead of changed in cross-sectional area. The variable in the redesign is the distance between the detecting electrodes. This distance can be related to axial force by means of calibration. Any axial force applied to the bag will increase the distance between the electrodes. The thick black and dark gray lines represents rigid plastic cylinders that ensures that the construction will not bend. The design also enables the bag to be inflated *in vivo*.

Axial force, based on strain gauge or impedance, has some limitations. Despite anchoring the probe to the nose or cheek, some movement will occur. This will affect the axial force recordings but in manometry would merely relocate the recording site. This movement will primarily influence the amplitude of the axial force recordings.

Future development of axial force recordings may include multiple recordings on the same probe. Recordings at different sites would provide a more complete picture of esophageal transport function and axial forces.

AXIAL FORCE RECORDINGS IN HEALTHY SUBJECTS

The axial force can be divided into two main components; the "grip" effect, and the ability to push in the axial direction again an intra luminal object[13]. The "grip" effect is primarily determined by manometry as the squeeze effect. A better grip will decrease the chances for the peristaltic wave to slip over a given intraluminal object and, thereby, create a basis for a strong axial force. The grip effect is also determined by frictional force. Any amount and type of fluid will change the frictional forces between the probe/bag and the esophageal wall and, thereby, change the grip effect. It has been shown that a swallow of 10 mL of salad oil decreased the amplitude by more than 50% in subsequent swallows[11]. The influence of frictional forces is an issue that needs further investigation before a statement about their influence in real-life situations can be made.

The information gained from axial force recordings has differed depending on the design of the studies. As with manometry, the absolute axial force values in control subjects vary markedly[11]. The coefficient of variation has been reported to be in the same range

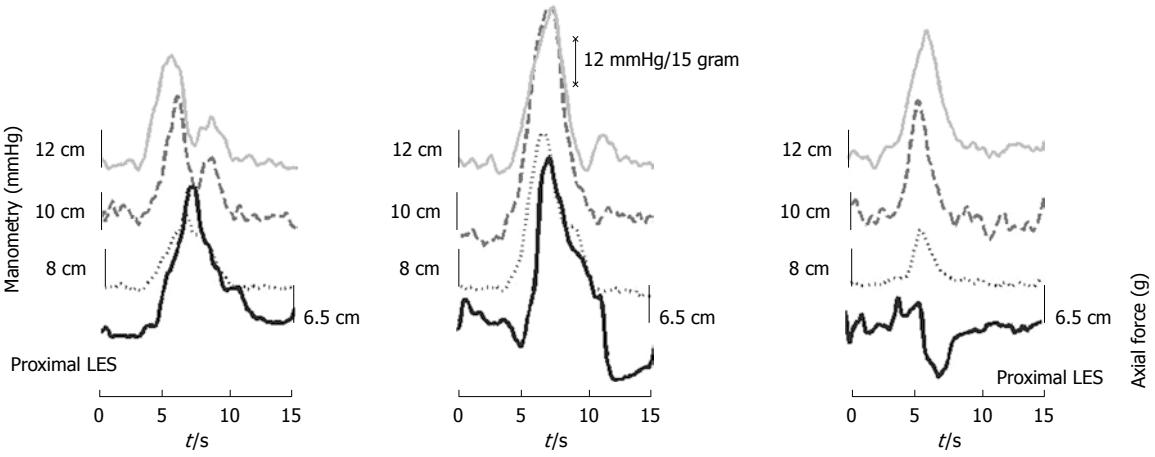

Figure 2 A voluntary dry swallow (time = 0) from three subjects with a bag volume of 2 mL. The solid black tracings are the axial force recorded 6.5 cm proximal to the lower esophageal sphincter, and the three other tracings are the pressure recorded 12 cm, 10 cm and 8 cm proximal to the lower esophageal sphincter. Using the manometric tracings, the first swallow (left) showed a normal propagating peristaltic wave and the resulting axial force response was as expected. The swallow of the second subject (middle tracings) shows a rather powerful pressure amplitude; but, it is not propagating (peak amplitudes are similar in time). In contrast, the axial force response is comparable in amplitude to that in the first subject (left tracings) although it is followed by axial force in the oral direction (negative value). In this case, the interpretation of the manometric recording would be flawed if the axial force had not been recorded simultaneously. The third person (right tracings) had lower manometric amplitudes during swallowing, without any propagation. The axial force response is different from the swallowing in the second person (middle tracings), as there is a weak reflux (oral axial force). The data shown is taken from a study in our group where the data is still being analyzed.

for manometry[22] and axial force[23]. These finding are similar to data collected in our laboratory. In assessing whether or not multiple manometric tracings along the esophagus are propagating, axial force recordings have shown to be inconsistent with the interpretation gained from manometric recordings[11]. This has also been documented using other modalities such as radiographic or scintigraphic imaging[21,24,25]. As an example from our laboratory, Figure 2 shows three dry swallows from three healthy subjects with a bag containing 2 mL saline. Three manometric tracings (12 cm, 10 cm and 8 cm proximal to the lower esophageal sphincter), and one axial force (6.5 cm proximal to the lower esophageal sphincter) tracings were recorded. These three examples show how the recordings can be very difficult to interpret when the bolus transit is deduced solely from manometry tracings.

Our probe design enables axial force recordings with various bag sizes to provide a challenge test for the esophagus (e.g. greater force with larger bag size). The effect of the bag size during force recordings was studied to some extent by Winship *et al*[12] who inflated a bag with 10 mL after which a swallow was initiated. The axial force applied to the bag from the peristaltic wave persisted until the bag was deflated. A comparable experiment was done by Pouderoux and co-workers using bag diameters of 15 mm to 20 mm[6]. During a study in our group a persisting axial force was occasionally generated at even smaller bag volumes (6 mL). However the data analysis for this study is not complete. Swallowing studies recording axial force have previously been performed with a balloon in the esophagus inflated with either air[11,12,14] or fluid[19]. Increased bag volume increased the axial force amplitude[11,14,15]. This could be due to an improved grip effect. These studies extracted parameters from axial force and manometric recordings and found little or weak correlation[6,11,13,14]. Thus, additional information is likely to be gained by recording axial force. Using clips attached to the esophageal mucosa, the shortening/elongation of the esophagus was measured in a study by Pouderoux and co-workers[6]. They combined shortening information with axial force and manometry recordings. A good correlation was found between axial force and (1) shortening of the distal esophagus, (2) the maximal contraction of the distal esophageal segment, and (3) the extent of aboral movement during the period of axial force recordings. These factors are all involved in the longitudinal muscle contraction. Hence, axial force recordings may show defects in this area where manometry provides poor information. Unpublished data from our laboratory on pressure and axial force recorded simultaneously have shown that the amplitude of manometry increases only slightly proximal to the bag (approximately 30%) when the volume is increased whereas axial force increased to a major degree (approximately 130%) (Figure 3). Somewhat similar findings were obtained in a previous study[11]. Axial force was also present with an empty bag. This could be due to the size of the probe (5 mm in diameter) as the grip effect is sufficient to generate an axial force. The amplitude for axial force, and manometry increased when dry and wet swallows were compared.

Using area-under-curve for manometry tracings as a parameter to predict the axial force response has also failed as there was very little correlation between this parameter and the area-under-curve for the axial force tracings[21].

The clinical standard procedure with manometry during swallow tests does not include a bag being inflated, as this only affects the manometric recordings to a minor degree[26,27]. On the other hand, increasing the bag volume would present a challenge test to the esophagus similar to an electrocardiogram recorded during exercise. During exercise, the electrocardiogram

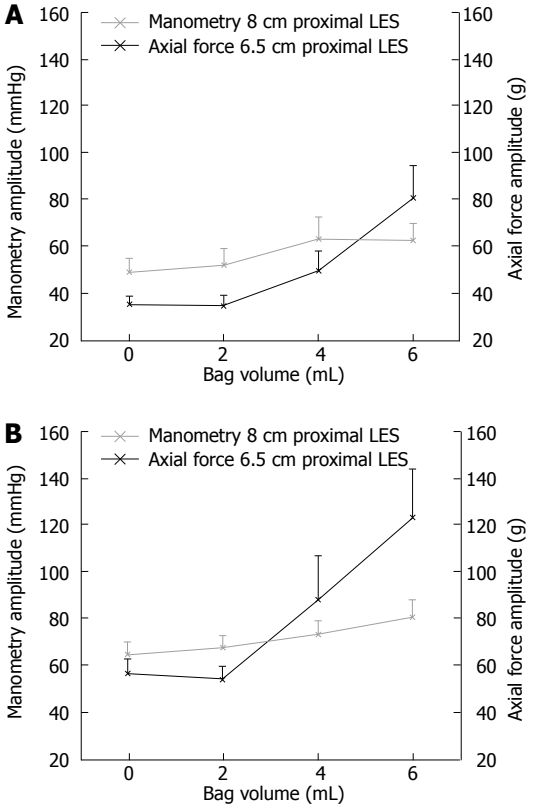

Figure 3 Swallow test with increased bag volume. A: Dry swallow; B: Wet swallow. The pressure was recorded 8 cm proximal to the lower esophageal sphincter and the axial force was recorded 6.5 cm proximal to the lower esophageal sphincter. Both graphs show that the increased amplitude for axial force was greater when compared to manometry during both dry and wet swallows. Data are presented as mean ± SE from 10 volunteers.

can reveal abnormalities not seen at rest[28]. Thus, a "challenge test" could provide a more sensitive test when recorded with axial force. This would be of great interest when applied to patients with motility-related diseases.

AXIAL FORCE RECORDINGS IN PATIENTS

Axial force is not a widely used technique and only a limited number of patients have been examined. In one study, a group of eight subjects complaining of dysphagia were examined. Radiography failed to reveal an organic narrowing of the esophagus, and no abnormal motor activity was observed by the fluoroscopist. Furthermore, the manometric examination did not reveal any dysfunction of peristalsis. On the other hand, axial force recordings clearly separated these subjects from healthy controls[11]. The patients were divided into two subgroups, one which had abnormally high amplitudes, and the other with abnormally low amplitudes. In 30 gastro-esophageal reflux patients with erosive disease and normal manometry, six patients showed impaired axial force amplitudes. This was based on a protocol with ten wet swallows (10 mL) for each patient. A similar pattern was shown in the same study where six out of twelve patients suffering from functional dysphasia had impaired axial force amplitudes but normal manometric amplitudes[17]. These results point in the direction of diagnosis being based on both manometry and axial force. If the result of multiple modalities can be provided by one examination, it will minimize the number of investigations and inconvenience for the patient without affecting the final diagnosis. Axial force has been measured simultaneously with manometry and, if not incorporated in the same catheter[11,13,19], manometry can be recorded next to the axial force probe[12,17]. The information gained from such investigations would be pressure and axial force generated by esophageal contractions. The preliminary results from studies in the last forty years have indicated that axial force recordings add further information to traditional manometry, and it remains unanswered why axial force is not more widely used.

CONCLUSION

Axial force measurement provides additional and more physiological information about the swallowing function compared to manometry. In future studies, combined axial force recordings and manometry may provide more useful information on esophageal muscle function in basic and clinical studies, especial protocols where a challenge test of the esophagus is incorporated will be of major interest in basic and clinical studies. It has been suggested that manometry could subgroup gastro-esophageal reflux disease patients into those requiring partial or total fundoplication treatment[29-31]. However, studies have not been able to prove that preoperative manometry could predict postoperative outcome in terms of dysphagia[32-34]. Measurement of axial force is more likely to identify patients who would develop postoperative dysphagia, although proof awaits clinical studies.

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GUIDELINES CLINICAL PRACTICE

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Do we really understand the role of the oesophagogastric junction in disease?

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Abstract

The role of the oesophago-gastric junction (OGJ) in gastro-oesophageal reflux disease is still not completely understood, and there is no clinically used method to assess the OGJ function in patients. Only indirect methods such as pH studies are carried out. The OGJ acts a valve controlling the flow of solids, liquids and gases between the oesophagus and the stomach. Manometry can determine if a sphincter is toned or relaxed; but, it cannot confirm that the sphincter region is actually open. Distension is a new technique for measuring function in the OGJ. By measuring the cross-sectional area through the narrow region in the junction during distension of a catheter mounted bag, much more information on the opening and closing patterns of the junction can be determined. This technique has already been demonstrated to show changes in the OGJ after surgical treatments for reflux disease. New measurement ideas around the concept of distending the OGJ offer new hope that a clinically useable test for compliance at the junction can be developed and could potentially help in determining appropriate therapy.

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Key words: Achalasia; Distensibility; Impedance Planimetry; Intraluminal impedance manometry; Oesophago-gastric junction

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INTRODUCTION

If you ask many clinicians, even those specialising in gastroenterology, what is the mechanism between the oesophagus and stomach which prevents the backflow of stomach contents into the oesophagus? Their instinctive reply will be the lower oesophageal sphincter (LOS). While the LOS certainly contributes to the mechanism it is not the sole effecter. Many common definitions of the mechanism have not even been updated. Medlineplus© defines gastroesophageal reflux disease as "backward flow of the gastric contents into the oesophagus due to improper functioning of a sphincter at the lower end of the oesophagus and resulting especially in heartburn[1]". The more clinical definition agreed at the Montreal Consensus does not make any mention of the relevance of the LES; "GORD is a condition which develops when reflux of stomach contents causes troublesome symptoms and/or complications". A definition very much based on effect rather than cause[2].

Of course with a little reading, most of us will know that the barrier at the oesophago-gastric junction (OGJ) is rather complex with a number of mechanisms impinging on it. Mittal and Balaban's New England Journal of Medicine article from 1997 highlights the fact that the lower end of the oesophagus is

guarded by an intrinsic smooth muscle called the lower oesophageal sphincter, an extrinsic skeletal muscle called the crural diaphragm, the intra-abdominal location of the LOS, integrity of the phrenoesophageal ligament, and maintenance of the acute angle of His promoting a "flap valve" function[3-5]. It has also been concluded that this structure is very sophisticated in the neurophysiologic control of opening, and its anatomical configuration, and with respect to dynamic changes in forces from active and passive muscle structures in this region of the body.

DISEASES RELATED TO OGJ FUNCTION

Gastro-oesophageal reflux disease

Gastro-oesophageal reflux disease (GORD) is one of the most common diseases in Western Civilisation. GORD can progress to severe complications if untreated. It is a highly prevalent disorder and affects 10%-20% of Western populations[6]. GORD originates from a disturbance in the structure and function of the LOS barrier. Anatomical or structural abnormalities occur often in the presence of a hiatal hernia and physiological predispositions from abnormal motor function of the LOS and oesophageal body. Dysfunctional oesophageal motility coupled with a weak LOS can cause uncoordinated propulsion of food, regurgitation of food and acid into the oesophagus, particularly after meals, and in the horizontal position, an inadequate acid/bile clearance from the oesophagus.

Achalasia

Achalasia is a motor disorder of the oesophagus characterized by loss of oesophageal peristalsis and failure of the LOS to relax completely upon deglutition. There is a poor correlation between higher sphincter pressures, oesophageal emptying and achalasia symptoms in general[7]. The origin of achalasia is still poorly understood. There is evidence to implicate familial, autoimmune, infectious or environmental causes. Currently, it is not possible to determine how the OGJ or the sphincter function changes as the disease develops.

DIAGNOSIS OF OGJ RELATED DISEASES

By far the most common OGJ disorder is GORD. For most people, GORD is diagnosed by symptoms usually at a visit to their general physician or community doctor. However, for many people, pharmaceutical treatment of GORD does not completely eradicate their symptoms, and they must be referred to a gastroenterologist for further evaluation and treatment. Usually, the first test a gastroenterologist will carry out is an upper gastrointestinal endoscopy. This visual test will quickly identify the presence of any erosive disease in the region of the OGJ, and if there are visible signs of this erosion or Barrett's metaplasia.

These referrals may also undergo other tests if endoscopy is negative. Traditionally, this involved oesophageal manometry to evaluate peristalsis and to confirm sphincter position and activity. This is followed by a 24 h pH study using a catheter based system that can incorporate impedance monitoring or a catheter-free telemetry-type system. Intraluminal impedance is increasingly being used to determine whether non-acid reflux may play a role in patients with PPI-resistant reflux symptoms, chronic unexplained cough, excessive belching, and rumination[8].

Despite the important role the OGJ plays in reflux disease, apart from standard manometry[9], techniques have not focused on objectively measuring its function.

ROLE PLAYED BY OGJ

Even with a number of new tests for reflux disease, it is still difficult to determine the actual role of the OGJ in this disease. Of course, the only test which directly measures some aspects of the junction is manometry, and this has been shown, for some time now, not to be a reliable predictor of GERD on its own[10]. There has been little emphasis on assessing the mechanical properties of OGJ opening, and little is known about the dynamics of retrograde flow across the OGJ. Thus, it is time to revisit this and look at new evidence.

DO WE HAVE THE COMPLETE PICTURE?

If the OGJ has a role in digestive disease, and if none of the parameters we use to determine the severity of a patient's reflux disease relate to any direct objective and quantitative parameter or parameters, do we really have the complete picture? Should more research not be carried out into methods to determine the role of the OGJ in disease? If pH studies determine that there is too much acid refluxing into the oesophagus, can we always be confident that this is due to dysfunction of the LOS? Could it be some other mechanism in the process of digestion, such as peristaltic dysfunction and poor oesophageal acid clearance which is malfunctioning and causing the disease?

THE OGJ AS A VALVE

If we accept that the main action or combined actions at the OGJ is to narrow and close the junction between the stomach and the oesophagus, then we must accept that it works in a similar way to a valve. The medical dictionary defines a valve as "a bodily structure that closes temporarily a passage or orifice or permits movement of fluid in one direction only" (Merriam-Webster Online Dictionary copyright© 2005 by Merriam-Webster, Incorporated). In the context of the OGJ, this definition is quite limiting, and I prefer the more general web definition of "A valve is a device that regulates the flow of substances (either gases, fluidized solids, slurries, or liquids) by opening, closing, or partially obstructing various passageways[11]". The OGJ function is probably better described in this way. It is as much a control valve as an "on" and "off" valve. While food boluses and liquids travel antegrade, air and on occasion small

amounts of stomach contents must travel retrograde back up the oesophagus for venting purposes.

HOW CAN WE MEASURE VALVE FUNCTION?

This, in effect, can be a difficult question. In engineering, we can easily measure the performance of an on/off valve by checking it visually. If the open valve condition is created, it is usually possible to observe flow through the valve. In the closed valve condition, it can normally be inspected for leaks or other forms of dysfunction. If the valve controls flow rather than shutting off completely, then normally there will be a device included to determine the flow.

Obviously the situation is much different in the OGJ. Much of the anatomical structure in the region is soft tissue. This does not image well radiologically, although historically barium studies have given a good, but subjective indication of flow during swallowing. The OGJ tends to be very dynamic, and so does not image well using magnetic resonance. In addition, the image resolution of current MR technology and CT is of insufficient quality to be satisfactory.

Historically, the LOS has been evaluated using manometry which uses pressures measured at precise points on a luminal catheter to determine the forces applied by the squeezing or pushing of the sphincteric regions in luminal organs. Manometry has evolved into a very sophisticated procedure for determining pressure changes in luminal organs during motility, but it is no longer recommended for the diagnosis of reflux disease[10].

FUNCTIONAL TESTING FOR DIAGNOSIS

The flow diagram in Figure 1 outlines the pathway for a patient with gastro-oesophageal reflux disease. In up to 20% of cases, there can be a negative or non-optimal response to drug therapies such as PPIs or H_2 blockers. These patients are usually referred to a gastroenterologist or in some cases directly to a GI surgeon where the severity of their condition is evaluated.

Referred patients will most often undergo an upper GI endoscopy so that erosive signs of the disease or Barrett's metaplasia can be excluded. However, up to 80% of patients will have a normal endoscopy, and will be referred for oesophageal function testing[12]. Most of these patients will undergo pH and manometry studies. Manometry can be utilized to document landmarks for placement of the pH electrode. The older manometry method is to perform a catheter pull-through study which will identify the respiratory inversion point and establish the high pressure zone at the LOS. This would also then indicate the position of the z-line and determine the position of the 24 h pH catheter, usually 6 cm above the z-line or 5 cm above the proximal margin of the LOS. The 24 h pH study is then used to determine the total time the pH < 4 or a composite

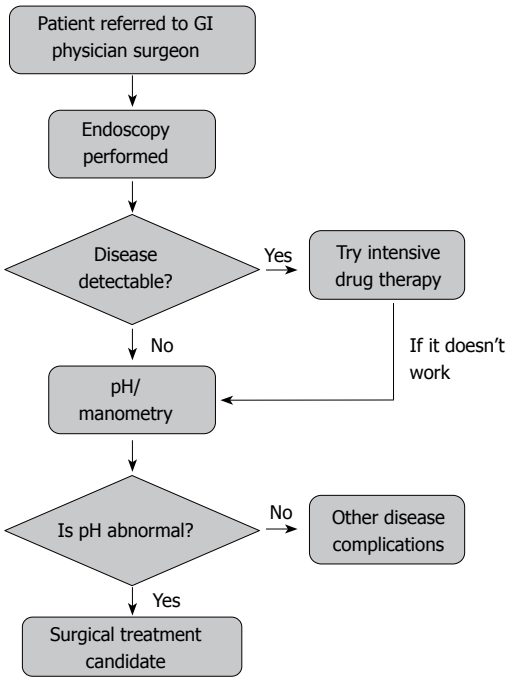

Figure 1 The pathway for a referred patient with gastro-oesophageal reflux disease.

score of oesophageal acid exposure. The DeMeester score uses 6 parameters (supine reflux, upright reflux, total reflux, number of episodes, number of episodes longer than 5 min, and the longest episode) to calculate a score which indicates the severity of reflux.

Newer methods for determining oesophageal function include high-resolution manometry (HRM), intraluminal impedance and wireless pH measurement. HRM is a method which uses sensors at 12 radial orientations and 36 longitudinal positions (1 cm intervals). In effect, this sophisticated probe can monitor the dynamic activity throughout the oesophagus from above the upper oesophageal sphincter to below the LES. Recent clinical evaluation has shown two major strengths of pressure topography plots compared with conventional manometric recordings. These strengths are the ability to (1) delineate the spatial limits, vigor, and integrity of individual contractile segments along the oesophagus and (2) to distinguish between loci of compartmentalized intra-oesophageal pressurization and rapidly propagated contractions[13].

In terms of reflux monitoring, the main advantage of the wireless pH system is the ability to attach a capsule to the inner mucosal surface of the oesophagus, thus negating the need to run an uncomfortable catheter through the patient's nasal passage[14].

The recent development of intra-luminal impedance as a measurement technique has added another option to the clinician's diagnostic armoury. This technique has been shown by numerous studies to be a sensitive and reliable means of detecting fluid or gas movement within the oesophagus. Hence it is a useful tool for determining the presence and extent of acid and non-acid gastro-oesophageal reflux, but not the quantity[15-17]. Despite these sophisticated tests none can measure the objective

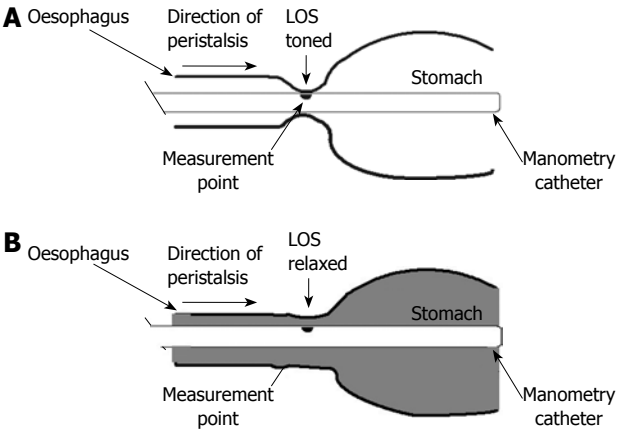

Figure 2 Simple manometry catheter shown in position in the LOS when it is toned. A: Manometry catheter with the black dot on the catheter indicating the measurement point in the oesophago-gastric junction when a normal LOS is toned or competent causing the sensor to be engulfed or occluded by the sphincter; B: The pressure is indicative of the state of the chamber created by the lumen (area in grey) opening up between the oesophagus and stomach.

role of the valvular effect at the OGJ in oesophageal disease.

CAN MANOMETRY MEASURE OGJ FUNCTION?

Since the work of Ingelfinger, Code and Fyke in the 1950s, most of the evidence which identifies the OGJ as the prime suspect in reflux disease has been based on manometric studies[18,19].

From the basic pull-through techniques to the development of the sleeve sensor, manometry has been used to describe sphincteric action at the junction. However, all the evidence in recent years suggests that it is not the ideal measurement technique. Recently, the AGA has advised that manometry should not be used to verify the presence of reflux disease. Yet, it is often still used as a technique in research studies[10,20-22].

A manometer is actually an instrument designed for measuring gas pressure. We should also remember from physics that pressure is a measure of force per unit area. Oesophageal manometry is often incorrectly defined as the degree of pressure exerted by the muscles of the oesophageal wall. The less specific definition of manometry as a method of recording pressures within the oesophagus is probably more appropriate. Strictly speaking muscles do not exert a pressure, they exert a force or rather forces in several directions. It could be argued that a manometric system cannot measure these forces; however, what it can do is use pressure as a rough proxy.

Consider the case of a manometry sensor on a catheter type probe suitable for inserting into luminal organs such as the oesophagus. Whether it is a water perfusion 4 port catheter or a state-of-the-art 36 position, 12 radial sensor measuring high resolution manometry catheter, the sensing principle is the same. Figure 2 indicates how this concept of measuring pressure may

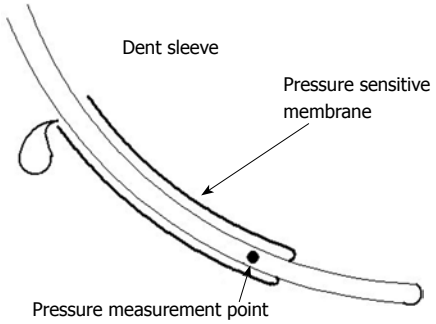

Figure 3 The dent sleeve.

be limiting. In Figure 2A, we can see the manometry catheter with the black dot on the catheter indicating the measurement point. This point represents a sensor on any manometry catheter. When a normal LOS is toned or competent, it exerts a squeeze or force on the sensor causing it to be engulfed or occluded by the sphincter. Hence, the pressure measured provides a reasonable representation of the force in the tightened sphincter. However, if the sphincter is relaxed, as demonstrated in Figure 2B, then rather than the lack of squeeze or force present being represented by no or low pressure at the sensor, the pressure here is more indicative of the state of the chamber created by the lumen opening up between the oesophagus and swallowing. Hence, the pressure observed at the measurement point is the force over a large area indicated by the shading in the diagram. We can conclude from this, when making manometric measurements in the LOS, that when the sphincter is in the toned state, we are actually measuring under a different set of parameters than when it is relaxed or not exerting a force on the pressure measuring sensor point. The measurement being made during swallowing is also altered by the peristaltic wavefront creating a bolus pressure that is pushing through the relaxed OGJ-highlighted bolus flow pressure and elastic properties of the OGJ.

One of the biggest developments in manometry was the introduction of the sleeve sensor in 1976[23]. Difficulties with reliable monitoring of maximal sphincter pressure with perfused side holes led to development of the Dent sleeve (Figure 3). The Dent sleeve recognized that, because of continuous movement within the lumen of the recording catheter and the narrowness of the region in the sphincter at peak pressure, it is impossible to guarantee that you are measuring the peak pressure continuously with a normal point measurement catheter. The sensing function of the sleeve ensures that it records maximal sphincter pressure, regardless of where the sphincter is positioned along the sleeve length. These unique recording properties make the sleeve the only sensor that can monitor sphincter pressure reliably using a static probe. However, new high-resolution manometry catheters are not limited by this movement artifact. The pressure drop measured by the Dent sleeve or high-resolution manometry is sometimes mistakenly assumed to be correlated with sphincter opening.

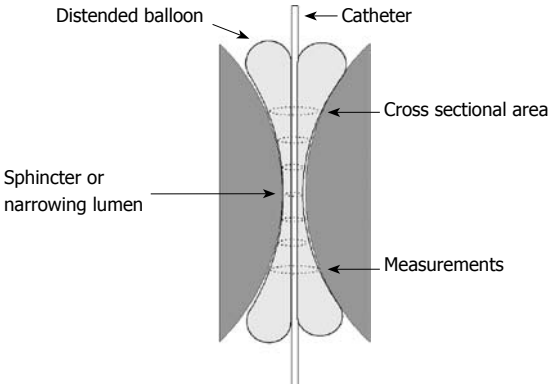

Figure 4 The functional luminal imaging probe (FLIP) showing how a cylindrical shaped bag mounted on the distal end of a catheter can use impedance planimetry to measure multiple cross-sectional areas (CSAs) at fixed intervals along a catheter.

DISTENSIBILITY AS A NEW MEASURE

Recently, a number of groups have been looking at the concept of distending the junction as a better measure of its performance. This suggestion is not a new idea. In fact as early as the 1960's, Harris and Pope identified that sphincters do not necessarily need to squeeze or contract tightly to be competent and, therefore, resistance to distension by measurement of radial force should be the prime determinant of sphincteric strength[24]. Studies on yield pressure in the stomach were probably an early distension method[25,26]. This involved filling air into the stomach and monitoring the pressure reading when the OGJ was forced to open to allow air to travel up into the oesophagus.

In 2002, Pandolfino and coworkers demonstrated that the OGJ of patients with hiatal hernias was more distensible and shorter than normal subjects[27]. This was done using manometry, fluoroscopy and stepwise, controlled barostatic distension of the OGJ. Then by controlling the pressure, the OGJ diameter and length were measured during deglutitive relaxation. In reality, it is difficult to control pressure in a bag located at the distal end of a narrow diameter catheter.

Shaker *et al*[28], demonstrated that under different conditions, the effects of muscle action and passive elastic tissue on the distensibility of the junction could be determined. The investigators used a barostat bag, cylindrical in shape, placed across the OGJ and recorded pressure, volume and distensibility as changes in pressure related to changes in volume. These changes were only a rough proxy for distensibility changes in the digestive lumen at the oesophago-gastric junction, since changes in the bag volume could be attributed to all forces on the bag, and not just those relating to valvular or sphincter action. Nevertheless Shaker *et al*[28] went on to show how this technique could indicate an increase in pressure at stepped bag volumes, and implied that this was related to a reduction in the distensibility of the OGJ. This work clearly demonstrates that a distensibility test may be useful in evaluating the effects of the different components on OGJ function.

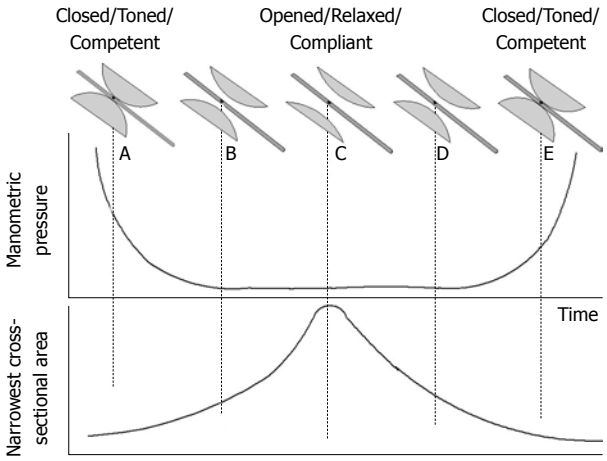

Figure 5 Diagram showing the difference between manometry and cross-sectional area (CSA) for measuring luminal changes in the oesophago-gastric junction during opening and closing. At point A where the junction is closed, there is a high pressure and a low CSA representing the toned sphincteric region. As the sphincter relaxes, very quickly the manometry catheter is no longer squeezed, and at point B the pressure is zero or very low. However, this opening is detected by an increase in CSA at B. At C the sphincter is fully relaxed; but, there is still no useful information from manometry despite the CSA increasing even further. The pressure does not start to rise again until the sphincter is fully closed and toned at E.

The functional lumen imaging probe (FLIP) takes distensibility testing to a higher level. FLIP allows a cylindrical shaped bag mounted on the distal end of a catheter to measure multiple cross-sectional areas (CSAs) at fixed intervals along the catheter. These CSAs can be used to build a dynamic three dimensional representation of the luminal geometric changes in the OGJ as the bag is distended on the catheter (Figure 4). In this way, luminal changes in the OGJ can be visually represented instantaneously, and with high spatial and temporal resolution[29,30]. This technique has been shown to demonstrate differences in patients undergoing endoluminal therapy for reflux[31].

MANOMETRY VERSUS DISTENSIBILITY

So what is the difference between measuring contraction or squeezing with manometry, and measuring opening or relaxation patterns using a distensibility technique? At the top of Figure 5 a simple series of drawings demonstrates the pattern of the sphincter part of the OGJ, and how it changes from being toned, compliant or competent to being relaxed or incompetent and then as it moves through the cycle back to being toned again. It can be seen how this cycle is measured using a manometry catheter or a distensibility probe such as FLIP, which can measure the narrowest CSA in the OGJ region. At the time point, represented by the dashed line A, the sphincter is contracted or squeezed, manometry will indicate by pressure that the force on the catheter is relatively high. Moving in time to the point at line B, it is evident that the sphincter has started to relax; the manometric pressure has dropped significantly since the sphincter no longer obscures or squeezes the pressure measuring point. Then, at time point C the sphincter

is fully relaxed or open; there is no significant change in the manometric pressure reading from time point B. However, the CSA value continues to increase to a maximum value. In practice, pressure can tell us the difference between a toned and a relaxed sphincter. CSA can tell us the difference between an open and closed sphincter. It can, in effect, tell us the amount of opening. As the sphincter starts to close and is no longer relaxed, it can be observed that manometry is very insensitive to this action at time point D, whereas CSA has detected the narrowing pattern. Then, eventually, at time point E, manometry has detected the squeezing effect of the toned sphincter, and the CSA has reduced to the smallest measurable value.

The most significant difference between manometry and distensibility is in the type of measurement. Manometry measures pressure which is a proxy of the combined active and passive forces during squeezing. Ideally, it does not interfere with the measurement. In reality, you cannot measure a toned sphincteric region using a pressure point on a catheter which is running through the sphincter without interfering with its function. Distensibility, on the other hand is more of a challenge test. By inflating the liquid filled bag as it straddles the OGJ, we are able to measure its response to ramped or stepped distension.

CONCLUSION

There are no available objective tests for testing OGJ function in routine clinical practice. Current gold standard tests for GORD or achalasia tell us very little about the role of the OGJ in disease. Much of the evidence for the role of the OGJ is related to manometric studies carried out more than 20 years ago. The experts advise us that manometric measurements should not be used to confirm GORD. Yet, most practitioners accept that there is a strong relationship between OGJ dysfunction and reflux disease. It is clear that manometry, while being a great tool for assessing motility, does not provide optimal information for OGJ evaluation.

A distensibility test using a bag catheter probe such as FLIP may provide better information on the opening and closing dynamics of the OGJ, rather than just relying on the sphincter tonic state as measured by manometry. New measurement ideas around the concept of distending the OGJ offer new hope that a clinically useable test for compliance at the junction can be developed and could potentially help in determining appropriate therapy.

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Sensory testing of the human gastrointestinal tract

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Abstract

The objective of this appraisal is to shed light on the various approaches to screen sensory information in the human gut. Understanding and characterization of sensory symptoms in gastrointestinal disorders is poor. Experimental methods allowing the investigator to control stimulus intensity and modality, as well as using validated methods for assessing sensory response have contributed to the understanding of pain mechanisms. Mechanical stimulation based on impedance planimetry allows direct recordings of luminal cross-sectional areas, and combined with ultrasound and magnetic resonance imaging, the contribution of different gut layers can be estimated. Electrical stimulation depolarizes free nerve endings non-selectively. Consequently, the stimulation paradigm (single, train, tetanic) influences the involved sensory nerves. Visual controlled electrical stimulation combines the probes with an endoscopic approach, which allows the investigator to inspect and obtain small biopsies from the stimulation site. Thermal stimulation (cold or warm) activates selectively mucosal receptors, and chemical substances such as acid and capsaicin (either alone or in combination) are used to evoke pain and sensitization. The possibility of multimodal (e.g. mechanical, electrical, thermal and chemical) stimulation in different gut segments has developed visceral pain research. The major advantage is involvement of distinctive receptors, various sensory nerves and different pain pathways mimicking clinical pain that favors investigation of central pain mechanisms involved in allodynia, hyperalgesia and referred pain. As impairment of descending control mechanisms partly underlies the pathogenesis in chronic pain, a cold pressor test that indirectly stimulates such control mechanisms can be added. Hence, the methods undoubtedly represent a major step forward in the future characterization and treatment of patients with various diseases of the gut, which provides knowledge to clinicians about the underlying symptoms and treatment of these patients.

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INTRODUCTION

Abdominal pain is very common in the general population[1], and pain is the most prevalent symptom in the gastrointestinal (GI) clinic[2]. Gastroenterologists face a challenge in treating these symptoms. Consequently, characterization of visceral pain is one of the most important issues in the diagnosis and assessment of organ dysfunction, as diseases giving rise to deep pain often are difficult to diagnose. This is partly due to the sparse and diffuse termination of visceral afferents in the spinal dorsal horn that overlap several segments, which is further complicated by convergence with somatic afferents (spinal convergence), autonomic (involvement of vagal nerve and spinal afferents) and enteric nervous (homeostatic and secretory) systems. Hence, activation of pe-

ripheral sensory afferents may lead to symptoms related to GI motor function (sweating, vasodilation, nausea and vomiting), which blurs the clinical picture. Consequently, complaints related to the autonomic nervous system or related to referred somatic pain are a clinical challenge. To understand the sensory system and how it can be tested, it is important to understand the basic neurophysiological mechanisms behind GI pain.

In healthy subjects, most visceral afferent activity does not reach higher brain centers, except information regarding filling of the esophagus, stomach, rectum and bladder. However, when the internal organs are potentially in danger - e.g. *via* inflammation and diseases - symptoms such as discomfort and pain are typically reported. The feeling is mostly vague and difficult to characterize, in contrast to distinct localization and characterization in somatic diseases. The different neuroanatomical structures of the two systems explain to some degree why visceral pain is more challenging to diagnose than its somatic counterpart (Figure 1). Visceral afferents that mediate conscious sensations run predominantly together with sympathetic nerves that reach the central nervous system (CNS), although some afferents join parasympathetic and parallel pathways. However, the upper esophagus and rectum also possess somatic innervation. The importance of this dual innervation is not clear, although the rectum has more complex functions than most other visceral organs and may need a more differentiated innervation. The peritoneum and parietal serous membranes of the lungs and heart possess their own parietal nerve supply, which is organized like the skin[3]. Hence, pain from these structures gives a distinct, intense and localized pain, which is comparable to the pain evoked by skin lesions. Most of the visceral afferents converge with lamina I , II and V spino-thalamic tract (STT) neurons, which receive input from both superficial and deep somatic tissue as well as other visceral organs[4]. Although the neuronal mechanisms are more complex, this convergence leads to referred somatic pain as well as viscero-visceral hyperalgesia. The latter phenomenon may explain several comorbid conditions such as increased number of anginal attacks in patients with gallbladder calcinosis, and increased number painful sensations to normal air and feces in the gut in patients primarily suffering from dysmenorrhea[5-9]. Most visceral organs exhibit spinal representation overlapping multiple segmental levels[10]. This widespread and low-density nature of visceral sensory innervation explains why large areas of the gut appear to be relatively insensitive to pain stimuli. The extensive resulting CNS activation may also explain the diffuse and unpleasant nature of visceral pain. Finally, unlike the somatic system, where prolonged or summated stimuli such as during inflammation are necessary to activate the N-methyl-D-aspartate (NMDA) receptor, it seems as though - in the visceral context - the NMDA receptor can be more easily activated by short-lasting and low intensity stimuli[11,12]. The resulting amplification of nociceptive processing explains why the manifestation of visceral pain is so often unpleasant and intense in its clinical presentation.

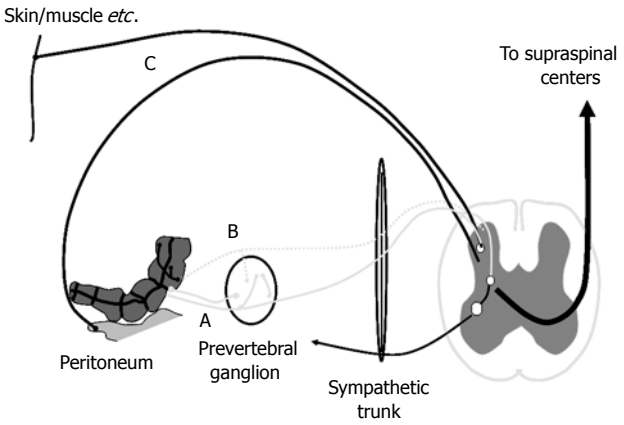

Figure 1 Afferent nerve supply of the gut. True visceral afferents innervate the gut, and most run temporarily together with either the sympathetic or parasympathetic nerves to enter the spinal cord. During inflammation, silent afferents (dashed line) may become activated and contribute to the sensory response. The peritoneum and parietal serous membranes of the lungs and heart have their own parietal nerve supply, which is organized like that of the somatic structures.

THE RATIONALE FOR EXPERIMENTAL STIMULATION OF THE HUMAN GUT

In clinical practice, several symptoms of underlying diseases confound the characterization of pain. These may include complaints relating to psychological, cognitive and social aspects of the illness, as well as systemic reactions such as fever and general malaise[13]. Furthermore, analgesic treatment and other medications often cause sedation and/or other side effects, which invariably bias the clinical evaluation of pain-related symptoms. Consequently, most studies evaluating drug efficacy in sensory functions of the gut have included a large number of patients. As a result of the above factors, together with the heterogeneity of the material, complicated statistical models have frequently been used - albeit often with equivocal effects. In the clinical situation, this is not a major problem. But, in assessment of analgesics in clinical trials, these confounders can easily invalidate the outcome.

However, in experimental pain models, the confounding factors can often be turned to advantages in the assessment of basic GI functions, mechanisms of disease and treatment efficacy. Under these circumstances, the investigator controls the experimentally induced pain (including the nature, location, intensity, frequency and duration of the stimulus), and provides quantitative measures of the psychophysical, behavioral or neurophysiological responses[13-15]. Different experimental animal models have been used in this context. The advantages of these models are obvious: neuronal activity can be studied directly in anesthetized or spinalized animals with invasive recording techniques or *via* assessment of behavior[16]. However, as neurobiology of the pain system differs substantially even between animal species, translation from animal studies to human pain studies has some major shortcomings.

Human experimental pain studies have for those rea-

sons gained much interest during recent years. In man, pain is closely related to culture, linguistic terms and expressions, and should be regarded as the net effect of complex multidimensional mechanisms that involve most parts of the CNS including intensity coding, affective, behavioral and cognitive components. This explains some of the difficulties and challenges in quantifying human sensory experiences with simple neurophysiological and/or behavioral methods, and why interest in more advanced human experimental pain studies has increased rapidly during the last decade[13,17]. The ultimate goal of advanced human experimental pain research is to obtain a better understanding of pain mechanisms involved in pain transduction, transmission and perception under normal and pathophysiological conditions, such as clinical pain. Obviously, the risk of perforation and other complications during invasive procedures limits the testing possibilities when stimulating the gut. As a result of these difficulties in accessing the GI tract, visceral experimental pain testing is far more resource-intensive and challenging than the more traditional somatic pain stimulations. As a result, most previous visceral studies have relied on relatively simple mechanical or electrical stimuli. These methods are easy to apply. But they have numerous limitations[13]. One should bear in mind that as pain is a multidimensional perception, the response to a single stimulus of a given modality only represents a limited fraction of the entire pain experience. Hence, the possibility of combining different methods to gut stimulation and induction of hyperalgesia will provide the possibility to more closely imitate the clinical situation, and provide extensive and differentiated information on the visceral nociceptive system[13,18]. Ideally, experimental stimuli to elicit gut pain in humans should be physiological, minimally invasive, reliable in test-retest experiments, and quantifiable. Preferably, the pain should mimic observations in diseased organs by inducing phenomena such as allodynia and hyperalgesia. Most experimental studies have been completed in functional diseases such as functional chest pain, non-ulcer dyspepsia and irritable bowel syndrome; but, to some degree organic diseases (e.g. ulcers, inflammatory bowel disease and chronic pancreatitis) have also been investigated[18-23]. The different types of stimulation (electrical, mechanical, thermal, chemical and ischemic) that evoke visceral pain in humans, as well as their limitations, have been described in detail previously[13,24]. In this review, we focus on novel developments regarding test systems that allow standardized stimulation of the GI tract and their applications.

MECHANICAL STIMULATION OF THE GUT

In the last decade, several studies have addressed the mechanical and sensory function of the GI tract by means of mechanical distension. Simple and physiological gut distension, such as ingestion of well-defined meals, may be useful in clinical studies[25]. Balloon or bag distension is, however, the favored method as the mechanical stimulation intensity is easier to control. Most recent studies have used the Barostat based on volume changes in an air-filled balloon kept at constant pressure levels, and several protocols and stimulation paradigms have been recommended for the Barostat, such as phasic and tonic distension. These stimulation paradigms have been thoroughly discussed, and will not be described here - for review see Whitehead *et al*[26] and van der Schaar *et al*[27]. The major advantages of the Barostat system and similar pressure-volume-based methods are the relatively low cost and the documented reproducibility between laboratories[28]. Furthermore, they are reliable and easy to use for routine purposes. Such systems have also been used for assessing sensory and pain thresholds, and under different conditions, attempts have been made to calculate the compliance and tension of the organs[26,27,29,30].

A major pitfall in early balloon distension studies was the use of latex balloons causing large errors because of latex deformability and lack of control of the stimulation field. Consequently, polyurethane or polyethylene bags are now recommended generally. There are, however, still several limitations and sources of error with systems based exclusively on volume and pressure. These include the fact that the data obtained must be corrected for compressibility of air as well as other major concerns, for further details see Drewes *et al*[31]. Basically, a common mistake in GI distension studies is to consider the mechanoreceptors as pressure, volume or tension receptors. In fact, the sensory rating may not be strictly related to pressure (or tension) during gut distension.

Circumferential strain or stress are more likely parameters correlating with receptor responses to stimulus intensity[32]. This is partly due to the fact that strain is a non-dimensional parameter independent of the geometry of the organ and directly associated with tissue deformation. In fact, recent studies have clearly demonstrated that circumferential strain is an important determinant of mechanoreceptor-mediated responses[33-36]. Correspondingly, studies providing tension calculations based on Barostat methods have shown conflicting results, e.g. in a recent study of the stomach, the estimated tension seemed to correlate with the sensation[29,37], whereas another study showed a high inter-individual variability in the sensation score to the applied tension, which suggests that factors other than wall tension influence the sensation[38]. However, uncertainties in the assumptions given above, and lack of adequate geometric and biomechanical considerations can also explain these findings[39].

Methods based on impedance planimetry allows recordings of luminal cross-sectional area directly and calculation of the radius in the distended GI segment[33,35,36,40-42]. As an example, a schematic drawing of the multimodal rectal probe is shown in Figure 2. Estimates of circumferential wall tension, stretch and strain based on measured radius are more accurate than estimates based on volume exclusively[32]. Findings during rectal distension support this theory, as stretch ratio at pain detection threshold produces an excellent intra-class coefficient of 0.98, both

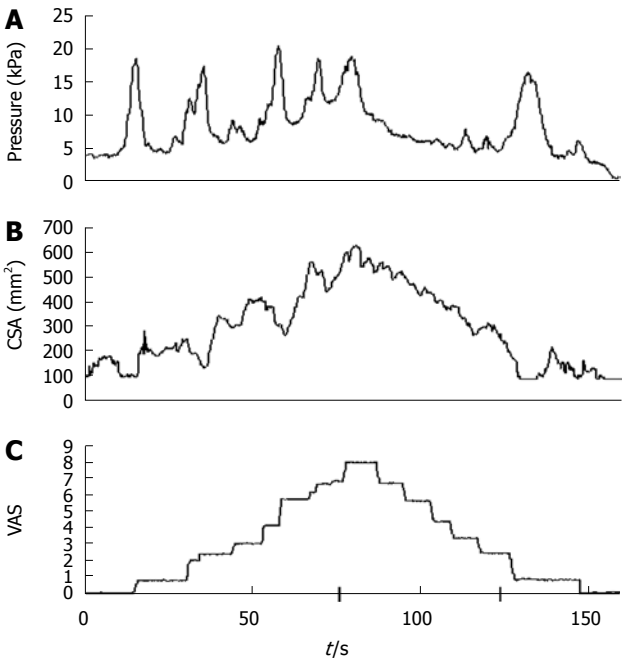

Figure 2 Illustration of mechanical stimulation in the esophagus. The bag was filled at an infusion speed of 25 mL/min. During distension, (A) pressure, (B) cross-sectional area (CSA) and (C) pain intensity was recorded on line. The increase in CSA corresponded with increasing stimulus intensity after the bag was filled with water, whereas there was little relation between the pressure waves and the sensation. The pain intensity was rated on a visual analogue scale (VAS), with 5 as the pain threshold. An intensity of 8 on the VAS resulted in reversal of the pump. For details see Drewes *et al*[35].

with and without administration of the antimuscarinic drug butylscopolamine[43]. An example of a rectum probe is shown in Figure 3. To reliably compute, e.g. rectal stress and strain, more complex modeling is necessary. Thus, in future studies mechanical distension combined with, for example, ultrasound methods or magnetic resonance imaging may offer the possibility of a better anatomical characterization of the GI tract[44,45].

ELECTRICAL STIMULATION OF THE GUT

Electrically induced depolarization of sensory afferents has been widely used as an experimental stimulus in humans. Electrical stimuli have proved to be safe in all parts of the GI system; however, it is recommended to monitor the heart during esophageal stimulation, as previous experiments have documented that atrial capture may occur[46].

Electrical stimulation of the GI tract has been used to study, for example, basic pain mechanisms[7,42,47-49] *via* evoked brain potentials to gut stimuli[50-52], and the effect of analgesics in both healthy volunteers and patients[53]. The main advantage of electrical stimulation is its reproducibility[43,54]. Also, its dynamic range (i.e. the range from sensation to pain threshold) is relatively high, which allows more robust assessment of sensory thresholds. A further advantage is that electrodes are easily implemented on different GI probes[13,43,54]. The well defined on- and offset of the stimulus makes it suitable to study pain mechanisms related to time, such as temporal summation[48,55] and cerebral evoked potentials.

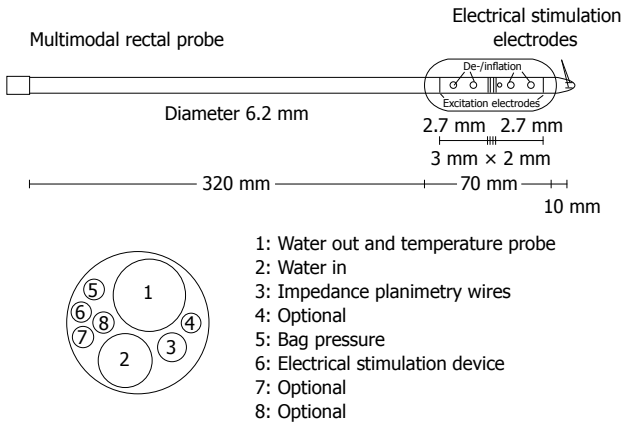

Figure 3 Probe used for measuring rectal CSA during distension.

There are, however, also limitations and drawbacks. Depending on the probe design and the electrodes, it may be difficult to obtain optimal mucosal contact between the electrodes and the GI tract because of, for example, longitudinal esophageal mucosal folds. Hence, it is necessary to measure and control the impedance between electrodes during stimulation, preferably at different frequencies. Further, electrical stimuli are neither natural nor specific for any sensory modality, and the electrical stimulation bypasses receptors, which stimulates all afferent nerves directly, including silent fibers. Consequently, electrical stimulation reflects the central nervous response rather than peripheral afferents. However, as most gut afferents are polymodal[56] and respond to a wide range of stimuli, specificity may be of minor importance. The electrical stimulation creates an electrical field, and the action potential is partly determined by the extracellular electrical potential, and partly by the nerve properties, including myelin and ion-channel configuration. Thus, there may be some selectivity relating to fiber type as the non-myelinated afferents (C-fibers) possess a higher activation threshold than myelinated fibers[17,57,58]. Hence, increasing stimulation intensity may depolarize myelinated fibers first, followed by C-fiber recruitment at higher stimulation intensities.

Electrodes can be either unipolar with a reference placed on the skin or bipolar with a set of electrodes. As a result of safety considerations, bipolar stimulation is recommended because the electrical field is more localized. Cardiac arrhythmia may theoretically be evoked by stimulation of nearby organs. Normally, atrial captures can be seen; but, this has no clinical significance. Arrhythmia can be avoided by either turning patch-electrodes away from the dorsal side of the heart, or by using bipolar ring electrodes that exhibit good mucosal contact[42]. Impedance should preferably be less than 3 kΩ before stimulation is initiated. Numerous stimulation paradigms have been recommended and no general consensus exists with respect to the configuration of the optimal electrical pulse. In fact, the stimulus should reflect the purpose, e.g. it is crucial to use single pulses in electrophysiological studies, where early peaks of evoked brain potentials are wanted. On the other hand, a single stimulus in the gut demands

Figure 4 An example of targeted colonic stimulation is shown, which demonstrates the electrode position. The controlled position, which can be altered in case of stimulation in vicinity of somatic structures and nerves, is a major advantage.

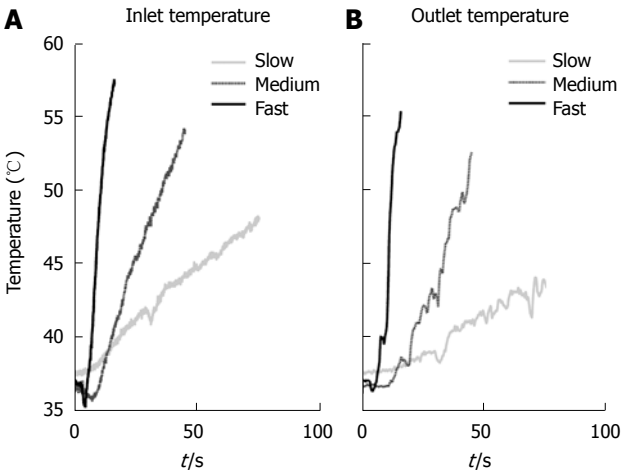

Figure 5 Temperature stimuli with three different incline rates. A: Left graph shows the temperature measured at the inlet of the bag; B: Right graph shows the temperature measured at the outlet of the bag.

rather high intensity to evoke pain, and trains or continuous series of pulses can be used in order to investigate temporal summation to a repeated series of stimuli (termed "wind-up" in animal experiments). Based on this experience, we use either: (1) single square pulses (duration 0.2-2 ms) in electrophysiological studies assessing evoked brain responses; (2) trains of five constant current pulses (rectangular with a duration of 1 ms applied at 200 Hz termed "single burst stimuli"), as such stimuli demand less current to evoke sensory responses; (3) "repeated burst stimuli" given as a series of single burst stimuli to investigate, for example, the central amplification of the repeated stimuli[48,55,59]; or (4) tetanic stimulation to be used for sensory thresholds of temporal summation[60]. This is applied as a train of pulses (0.2 ms, 100 Hz) that is linearly increased from zero. The advantage of this stimulation is that it is precise and less time-consuming.

Blind, untargeted stimulation is avoided by integrating electrodes onto endoscopic biopsy forceps, which makes stimulation of well-defined areas in the esophagus, stomach, duodenum, terminal ileum and colon possible[48,55]. An example of targeted colonic stimulation is shown in Figure 4. The major advantage of this modification is that electrode position is controllable and can be altered in case of stimulation in the vicinity of somatic structures and nerves. Further, mucosal contact is secured and evoked motor phenomena such as secondary contractions can be studied directly. Hence, the method allows characterization of local and referred pain to stimuli in most areas of the intestine relevant to localized pathology. However, the subjects have to cope with the rather thick endoscopes during experiments, which may be unpleasant especially in the upper GI tract, and which may cause bias in the pain assessment.

As gut segments exhibit differences in anatomy and innervation, a general consensus regarding stimulation location is warranted. Consequently, as described above, stimulation paradigms have a major influence on pain assessment. Thus, future studies should include standardized and validated optimal parameters such as stimulus duration, shape, polarity, frequency and intensity, which allows comparison between different laboratories.

THERMAL STIMULATION

In contrast to mechanical and electrical stimuli, thermal stimuli activate selectively, for example, being either mucosal heat-responsive TRPV1 receptors with temperatures above 43℃, or mucosal cold-responsive TRPA1 with temperatures less than 17℃[61]. Thermal stimulation has been used to study basic pain mechanisms[42,43,48,49,54,61,62], functional[63] and organic gut disorders[22] and analgesic efficacy in healthy volunteers and patients[53].

Rectal heat pain stimulation has been performed using a Peltier device[61]. To improve thermal stimulation in the gut, we have developed the multimodal esophageal probe - for details see Drewes and Gregersen[24]. Thermal stimulation is based on recirculation of cooled and/or heated water in the bag with a temperature sensor placed inside the bag. The method has also been integrated onto a multimodal rectal probe. In both cases, the most reliable proxy of the thermal energy applied is the area under the temperature curve[43,64]. In the esophagus, the method aims at having a constant high or low temperature in the bag until the pain threshold is reached. This method has been shown to be reliable and robust in drug experiments[54]. In studies of healthy subjects, it has shown some limitations, as not all subjects reach a pain threshold during the 2 min stimulation that were empirically found to be safe. To improve control over the stimulation intensity and duration, the method has recently been changed in order to obtain a linear increase in temperature, with an adjustable temperature ramp. Such stimulation is shown in Figure 5. In these experiments, the stimulation intensity can continue until the subject reaches the pain threshold, an improvement that is expected to result in better validity and reliability of the method.

CHEMICAL STIMULATION

Chemical stimulation of the GI tract resembles clinical inflammation and approaches the ideal experimental

visceral pain stimulus[7]. Such stimuli have successfully been applied to the skin[14,17,65] and muscles[66], but are also widely used in the gut. As an example, esophageal acidification is commonly used as a method to sensitize the gut evoking allodynia/hyperalgesia[67,68], but the model may also be used for direct stimulation[69]. The major relevance of the model may be induction of sensitization of visceral afferents to subsequent experimental stimulation. Chemical stimulation has been used to study, for example, basic pain mechanisms[42,43,48-50,62,70,71] and functional gut disorders[22,50]. However, drawbacks of chemical stimuli include a relative long latency time to onset of effects, and that the effects are often not reproducible[7]. Other stimuli such as glycerol, alcohol, bradykinin and other chemicals[72-75] have been used in uncontrolled studies, but their applicability has yet to be established. Recently, capsaicin has been used to evoke pain in the small and large intestine[76-78]. Chemical stimulation has also been used to explore basic functions such as autonomic changes in referred pain[79]. Hammer *et al*[77] have shown that activation of chemosensitive pathways induces symptoms that differ from those induced by mechanical activation, although animal data do not allow such a strict separation[80,81]. As a result of the relative inconsistency of the effects of acid perfusion in the esophagus[82], we recently used perfusion of a combination of acid and capsaicin in the human esophagus[83]. It is believed that capsaicin has an additional effect on acid because of synergistic mechanisms on the transient receptor potential vanilloid type 1 (TRPV1) channels[83]. The perfusion induced locally reproducible hyperalgesia to subsequent heat and electrical stimulation, and an expansion of referred pain in all subjects. The increased referred pain reflects convergence mechanisms on second-order neurons in the spinal cord, and can be used to elucidate the central component of hyperalgesia. A further step is achieved by the demonstration of viscero-visceral hyperalgesia in the rectum following esophageal perfusion with acid and capsaicin[84]. Hence, the model may be more robust than acid perfusion alone, but further studies are needed.

SPATIAL AND TEMPORAL SUMMATION

Lewis[85] has found that distension of the gut is most painful when long, continuous segments of the gut are distended simultaneously. Even greater pressures within smaller segments of the gut are not as efficacious in producing painful sensations. Hence, spatial summation is clearly an important contributor to visceral pain mechanisms. An experimental design to achieve spatial summation is mounting either multiple inflatable bags or one long bag on a probe, and then assessing the distension volume at pain detection threshold (PDT) derived from each bag, compared to the distension volume at PDT during simultaneous distension of multiple bags or the long bag.

Also, integration over time-temporal summation-is important. If electrical stimuli are repeated over time, both pain and the area of referred pain increase progressively[47]. The same phenomenon is seen following repeated distensions[7].

ACTIVATION OF INHIBITORY MECHANISMS

Pain inhibits pain, and impairment of descending control mechanisms is believed to be an important part of the pathogenesis of chronic pain[86]. Descending inhibition involves a spinal-supraspinal-spinal feedback mechanism, which results in direct or indirect inhibition of spinal neuronal responses[87,88]. Supraspinal sites can, however, also facilitate nociception, and the measured net output is either predominantly facilitation or inhibition[89]. Hence, experimental studies assessing human inhibitory processes measure the balance between these phenomena.

The most common method to provoke the noxious inhibitory system is the cold pressor test that is performed by immersing a hand or foot in 2.0 ± 0.3℃ ice-cooled water for at least 2 min. Efficacy of the descending control can then be investigated by comparing two stimuli separated by the cold pressor test. The cold pressor test has been used to study basic pain mechanisms and functional gut disorders[90].

MULTIMODAL APPROACH

Five to ten years ago, the available probes did not possess the ability to produce more than a few of the above-mentioned stimuli. Some authors have combined mechanical and electrical stimuli[91,92], and others have used electrical stimuli combined with sensitization to acid[50]. The esophageal multimodal probe and its use in basic, pharmacological and clinical studies has been reviewed recently, and the reader is referred to Drewes *et al*[18]. Recently, the model has been used in basic science, including other gut segments such as the duodenum[62] and rectum[43]. We are currently using the multimodal rectal approach in pharmacological and clinical studies of functional and organic disorders.

One limitation of multimodal pain stimulation in the gut is that it is done without visualization of the inside of the intestine. Hence, diseases such as esophageal erosions cannot be excluded. Recently, we have made an attempt to combine the multimodal probe with endoscopy, as illustrated in Figure 6, by passing a small (2.8 mm) video-endoscope into a multimodal probe, which allows mechanical, thermal and chemical stimulation. A schematic drawing of the probe is shown in Figure 6. As electrical stimulation can be done with electrodes attached at the biopsy forceps for the endoscope[48], the probe allows full multimodal stimulation including mucosal inspection and biopsies (albeit small) for histology and specific immunohistochemical staining. In diseases such as esophagitis, the TRPV1 receptor has been shown to be important[71] and the receptor also seems to play a role in sensation to heat and acid[93]. Hence, combined information about the sensory profile and

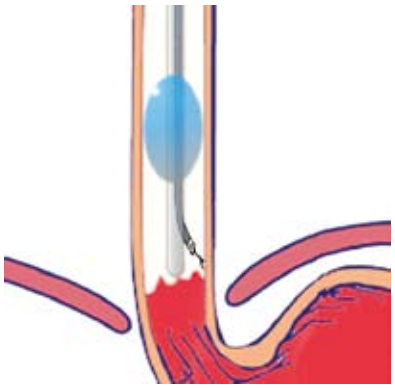

Figure 6 Newly developed multimodal endoscopic probe that allows comprehensive sensory information combined with visual mucosal inspection and biopsy specimens.

histological findings may be important in evaluation of the pathogenesis in diseases. Theoretically, the endoscope can be replaced with an ultrasound probe that allows assessment of the gut wall and, therefore, can be used for advanced mechanical modeling[94].

CONCLUSION

Over the last few years, the technical limitations of sensory testing in the GI tract have been increasingly surmounted. Multimodal esophageal, duodenal and rectal probes have been developed, which allow the investigator to use different stimulus modalities in the gut. The probes have proved to be robust across sessions, and have shown high reproducibility in all modalities. Future experiments using experimental testing will undoubtedly shed light on the pathogenesis of GI disorders, as well as assisting in finding new treatment modalities.

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GUIDELINES CLINICAL PRACTICE

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Imaging of the gastrointestinal tract-novel technologies

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Abstract

Imaging of the gastrointestinal tract is very useful for research and clinical studies of patients with symptoms arising from the gastrointestinal tract and in visualising anatomy and pathology. Traditional radiological techniques played a leading role in such studies for a long time. However, advances in non-invasive modalities including ultrasound (US), computed tomography (CT), positron emission tomography (PET), magnetic resonance imaging (MRI), *etc*, have in the last decades revolutionised the way in which the gastrointestinal tract is studied. The resolution of imaging data is constantly being improved and 3D acquisition, tools for filtering, enhancement, segmentation and tissue classification are continually being developed. Additional co-registration techniques allow multimodal data acquisition with improved classification of tissue pathology. Furthermore, new functional imaging techniques have become available. Altogether, the future of gastrointestinal imaging looks very promising which will be of great benefit in clinical and research studies of gastrointestinal diseases. The purpose of this review is to highlight the capabilities of the newest techniques to explore the detailed morphology, biomechanical properties, function and pathology of the gastrointestinal tract.

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Key words: Computed tomography; Gastrointestinal tract; Imaging; Magnetic resonance; Radiology; Ultrasound

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INTRODUCTION

Examinations with visualisation of the anatomy and pathology of the gastrointestinal (GI) tract are often mandatory in the diagnosis of GI diseases. For this purpose, traditional radiological techniques played a leading role for a long time. However, improvements in endoscopic examinations, the latest including wireless capsule endoscopy, have radically changed the possibilities for direct visualisation and intervention in the GI tract. The introduction and advances in non-invasive imaging modalities including ultrasound (US), computed tomography (CT), positron emission tomography (PET) and magnetic resonance imaging (MRI) have in the last decades revolutionised the way in which the GI tract is studied[1]. The resolution of imaging data is constantly being improved and 3D acquisition, tools for filtering, enhancement, segmentation and tissue classification are continually being developed. Additional co-registration techniques allow multimodal data acquisition (PET-CT, MR-PET, CT-US, *etc*) with improved classification of tissue pathology. Each modality is characterised by a distinct profile of favourable and unfavourable features, and the technique used depends upon availability, accuracy, usefulness, safety and costs. The diagnostic performance in terms of sensitivity, specificity and accuracy depends on several factors: the specific method and equipment used, the part of the GI tract investigated, patient constitution and preparation, most importantly the sort

of pathology being studied, and not least which "gold standard" the method is being compared to.

The purpose of this review is to highlight the capabilities of the newest imaging techniques to explore the detailed morphology, biomechanical properties, function and pathology of the GI tract. Table 1 provides an overview of the advantages and shortcomings of the most frequently used modalities in the study of the GI tract.

CONVENTIONAL RADIOLOGICAL EXAMINATIONS

Using non-contrast radiography, normal GI segments with no or little gas content cannot be separately visualised; but normal and abnormal gas accumulations, air-fluid levels, calcifications and motility of air contained in the intestines can be visualised[2].

In mono-contrast examinations, the intestinal lumen is filled with a positive contrast material in order to visualise peristalsis, emptying and pathological changes such as stenosis, dilatation, luminal filling defects and external compression. In double contrast examinations, the inner surface is coated with contrast material and the lumen is distended with air. This allows detailed visualisation of the mucosa which is especially useful in the detection of inflammatory and neoplastic changes of the small and large intestine[2]. However, the methods do not allow direct description of the deeper wall layers and extraintestinal lesions.

ANGIOGRAPHY

Conventional angiography of the GI tract has a clear role in the visualisation and treatment of GI bleeding. However, oesophago-gastroduodenoscopy and colonoscopy are the primary methods for identifying GI bleeding; but, sometimes these approaches cannot identify the source of bleeding[3]. In these cases, the leakage may be visualised using scintigraphy with tagged red blood cells, capsule endoscopy, double balloon endoscopy and increasingly multi-detector CT (MDCT)[3,4].

ULTRASONOGRAPHY

Transabdominal US is a safe procedure without any radiation exposure and allows visualisation of the intestinal wall, fluid-filled intestinal segments and the surrounding environment. US has excellent soft tissue imaging capabilities which make it ideal for both clinical and research studies of the GI tract. This is especially valuable in the detection of GI tract inflammation, where wall thickening, disturbed wall morphology, surrounding oedema and lymphadenopathy can be visualised[5]. In cases of extraintestinal fluid collections and abscess formation, mini-invasive drainage of these collections can be performed guided by US. Endosonography using intraluminal probes allow high-resolution imaging of the wall layers[6-8]. By applying special techniques (see below), additional information can be obtained.

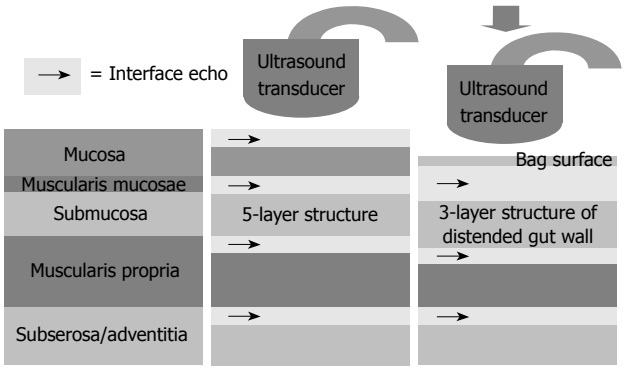

Figure 1 The principles of endosonography. The histological gastrointestinal wall layers (left) are correlated to the typical layered ultrasound appearance of the gastrointestinal wall (middle). The 5-layered appearance is due to the addition of several interface echoes at the tissue interfaces. During compression or distension the wall is further stretched (including mucosal unfolding) which together usually obscures the second echo-rich mucosal layer. Hence, the wall appears 3-layered (see Figure 2).

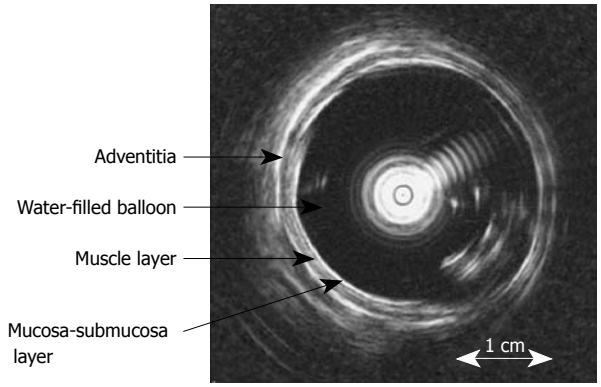

Figure 2 Cross-sectional endosonographic image of the distended distal oesophagus allows identification of three oesophageal sub-layers, i.e. mucosa-submucosa, muscle and adventitia. The white shadows inside the water-filled bag (4-5 o'clock) represent artefacts due to convulsions of the water-filled balloon, which results in reduced image quality at low degrees of distension. Modified from [17].

Perfusion of the intestinal wall and surrounding tissues can be assessed using Doppler imaging. Recently, the application of intravenous US contrast agents has improved the detection of hypervascularisation and hyperemia, especially in inflammatory bowel diseases[9,10].

Qualitative and quantitative information of intestinal motility and gastric filling/emptying can be obtained using transabdominal US[11,12]. 3D position and orientation systems allow real-time 3D visualisation with reconstructions and volumetry of the GI tract[13,14]. Intraluminal flow can be assessed using Doppler flow imaging[15]. This is especially useful in studying flow through the pylorus.

GI wall layers can be visualised endoscopically using high-frequency endosonography. This is normally used in tumour diagnosis, but has also been used experimentally to study e.g. the biomechanical properties of the GI tract[16,17]. Usually 3-7 layers of the wall can be visualised (Figures 1 and 2)[7]. The separate layers are bound together and possess dissimilar active and passive biomechanical (i.e. anisotropic) properties

Table 1 Most frequently used imaging modalities in the study of the gastrointestinal tract: Overview of main advantages and shortcomings

Modality	Advantages	Shortcomings
Multidetector computed tomography (MDCT)	High temporal and spatial resolution Fast image acquisition without motion artefacts Total evaluation of entire intestine and its surroundings 3D reconstructions and virtual endoscopy Possibility for image guided intervention	High radiation exposure Less suitable for research in healthy subjects No direct functional information Low risk of nephropathy due to intravenous iodised contrast media
Ultrasound (US)	High soft tissue resolution No radiation exposure Ideal for repeated examination and research Evaluation of intestinal wall and surroundings Information on motility, function and flow directly available using special techniques Possibility for intraluminal imaging Ideal for image guided intervention	Relatively high interobserver variability Intestinal gas lowers image quality Artifacts may be difficult to interpret Total visualisation of the entire intestine is difficult
Magnetic resonance imaging (MRI)	Good soft tissue imaging capabilities No radiation exposure Ideal for repeated examinations and research Total evaluation of entire intestine and its surroundings Functional and motility information directly available using special techniques	Motion artifacts due to intestinal motility Long image acquisition Image resolution less than CT making 3D reconstructions and virtual endoscopy cumbersome Potential long term effects of gadolinium-based contrast media (nephrogenic systemic fibrosis)
Conventional radiography	High temporal and spatial resolution Fast image acquisition Motility and function easily studied using intraluminal contrast	Only direct visualisation of luminal/mucosal properties Radiation exposure No 3D image data
Endoscopy	Direct visualisation of the mucosa Possibility for intervention (biopsies, polypectomy and endoscopic surgery)	Invasive procedure Discomfort and potential intestinal perforation No visualisation of deeper wall layers and surroundings

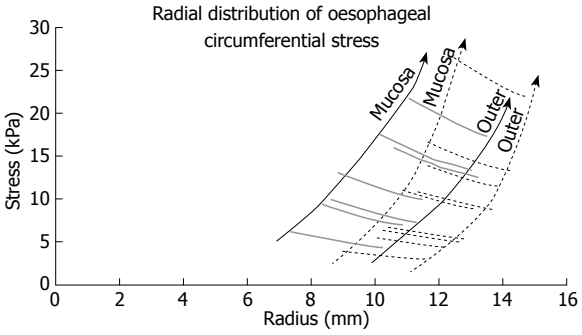

Figure 3 The oesophageal stress is calculated based on endosonography images and manometry. The alignment of solid curves represents the oesophageal stress profiles during oesophageal distension in a healthy volunteer. The alignment of dashed curves represents distension during butylscopolamine smooth muscle relaxation. As the oesophagus distends the inner radius and stress increases, i.e. the left end of the curves shift to the right and upwards. At high degrees of distension the steepness of the stress profile increases. Oesophageal relaxation shifts the alignment of the stress profiles to the right. Modified from [17].

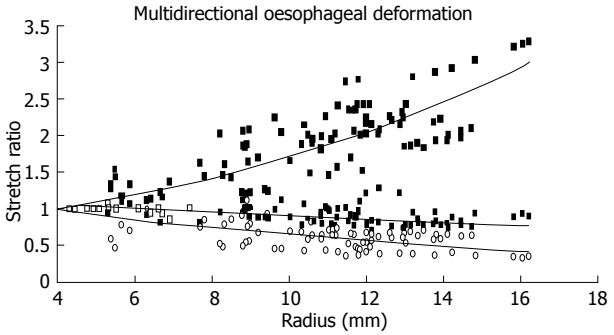

Figure 4 The circumferential, radial and longitudinal deformation of the oesophageal muscle layer is here described as the stretch ratio and as a function of the radius. The stretch ratio and radius are calculated based on endosonography. Data are from 12 healthy volunteers and shows a tensile circumferential stretch, radial compression and longitudinal shortening. Modified from [17].

which make a detailed analysis complex[16]. To assess the biomechanical properties, the intestine can be distended with fluid-filled balloons containing pressure measurement and US mini-probes providing cross-sectional images of the intestine[17,18]. The applied load on the wall can be controlled and accurately quantified. This allows calculation of passive and active biomechanical properties of the distended segment with parameters such as strain (relative deformation), tension, stress (force per cross sectional area) and stiffness of the wall layers[16,17,19]. The radial distribution of the circumferential wall stress has been assessed in the oesophagus[17]. Both the circumferential strain and stress were highest at the mucosal surface and decreased throughout the wall (Figure 3). The stiffness increased throughout the wall and was highest at the outer surface. The high stiffness of the muscle layers (compared to the mucosa) may limit the total oesophageal deformation (i.e. further distension) and protect the vulnerable and less stiff mucosa from damage when overstretched. This method has also been used to assess the multi-directional deformation and wall layer thicknesses of the oesophagus. Distension induces tensile circumferential stretch, radial compression and longitudinal shortening (Figure 4), which is not taken into account when using conventional barostat methods. This has shown to be valuable in the description of structural remodelling in organic GI disorders. Patients with longstanding diabetes

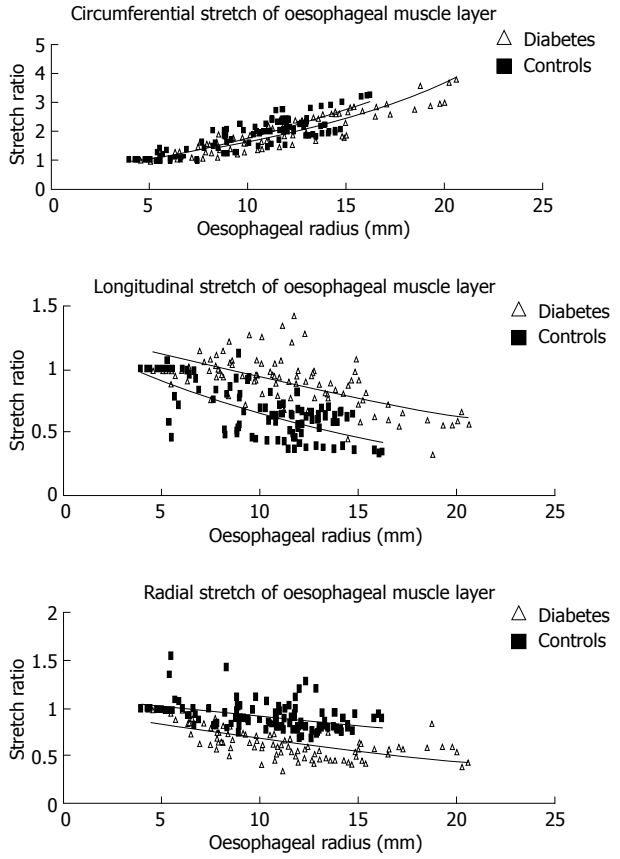

Figure 5 The graphs show the effect of diabetes on deformation of the oesophagus. The distension-induced change in oesophageal circumferential, longitudinal and radial deformation (stretch ratio) are calculated based on endosonography and illustrated as a function of the oesophageal radius. The curves were obtained during smooth muscle relaxation with butylscopolamine. The data points represent multiple measuring points during distension in diabetic patients and controls. Exponential trend lines (solid lines) of the diabetic patients and controls are shown. Oesophageal shortening during distension was clearly reduced in the diabetic patients while the radial stretch was decreased. Modified from [20].

mellitus have increased thickness of the oesophageal wall layers[20]. Also, longitudinal shortening was decreased in the diabetic oesophagus combined with a decreased radial stretch (Figure 5)[20]. Together with these structural changes indicating remodelling of the GI tract, diabetic patients also have an increased reactivity to oesophageal distensions and impaired coordination of the contractions which may reflect neuronal abnormalities due to autonomic neuropathy[20].

Further developments in the Doppler imaging technique can, when applied on the wall tissue itself, give information about movements inside the wall structure. This technique is known as strain rate imaging (SRI) and allows detailed mapping of the deformation of the wall layers with description of local tissue velocities[21]. Hence, the different contractile activity of the circular and longitudinal muscle layers can be visualised[12]. Elastography is a new US method which allows assessment of tissue stiffness. The tissue is compressed and the deformation pattern is visualised as colours (from soft to stiff) on the US image. The stiffness depends on the biomechanical properties, allowing differentiation between normal and abnormal tissues[12,22].

Cross-sectional endosonography of oesophageal contractions has shown that the cross-sectional area of the outer longitudinal muscle layer increases during contractions[23]. This indicates a contraction of the longitudinal muscle and shortening of the oesophagus which is thought to support the peristaltic force generated by the inner circular muscle[23]. Endosonographic studies have revealed that episodes of oesophageal chest pain and heartburn are associated with sustained contraction of the longitudinal muscle layer[24].

CT

The introduction of multidetector CT (MDCT) scanners with typically 64 detectors or more allows fast acquisition of thin slices and allows multi-planar reconstructions in any direction. This is a valuable tool in the study of intestinal loops[25]. Non-contrast enhanced CT scanning is increasingly replacing plain radiography in the evaluation of free intraabdominal air and intestinal obstruction. Intravenous contrast enhancement and filling of the intestinal lumen with water or positive contrast agents are performed in order to optimise imaging of the bowel wall. This is particularly valuable in the detection of inflammatory and neoplastic intestinal lesions, and allows accurate detection of extra-intestinal findings[26].

MDCT colonography is a relative new way of studying the large intestine. After proper colonic preparation, the large intestine is distended with air and the patient is scanned in the prone and supine positions[27]. The examination is reviewed in multiplanar views and as virtual endoscopy allowing flight-through of the intestine in both directions. This allows detection of smaller (> 6 mm) colonic polyps with a similar high accuracy to that of conventional colonography, while the accuracy for even smaller polyps is poor[27-29]. New generations of software with virtual dissection and unfolding of the colon will, together with computer-aided detection (CAD), probably improve the diagnostic accuracy and reduce the imaging time[30,31]. In addition, the detection of any incidental extra-colonic pathology is possible[32,33]. This technique may replace the traditional double contrast examinations in the case of incomplete colonoscopy and may also play a central role as a non-invasive screening examination.

MAGNETIC RESONANCE IMAGING (MRI)

MRI has no known short- or long-term hazards, and, therefore, provides excellent soft tissue imaging capabilities for studying the GI tract. This makes MRI favourable compared with CT which has considerable radiation exposure. However, intestinal MRI is limited by long acquisition times and a high risk of motion artefacts. Since the early days of MRI, the technology has advanced significantly and recent developments in MRI techniques such as parallel imaging, allow much faster and higher quality image acquisition.

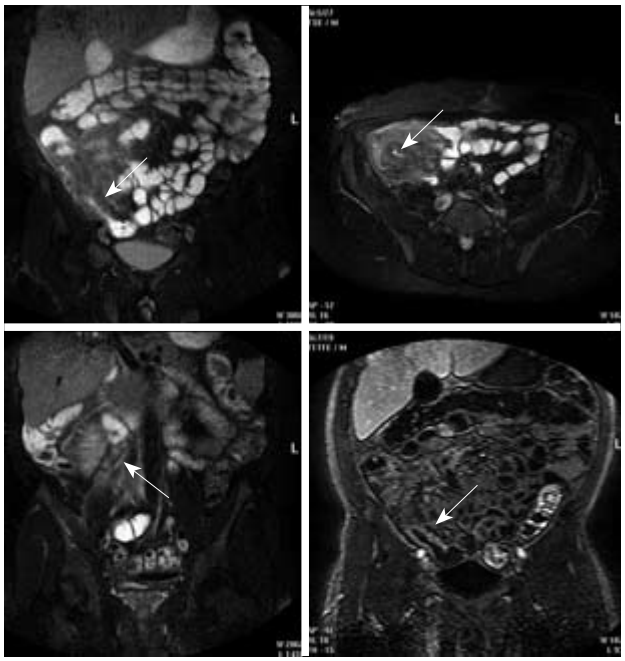

Figure 6 MRI of Crohn's disease. The coronal and axial (upper panel) fat-saturated T2-weighted MRI display marked wall thickening, mucosal irregularity and stenosis of the terminal ileum (arrows). Advanced mesenteric inflammation with hypervascularity and enlarged lymph nodes (arrow) are visualised on coronal fat-saturated T2-weighted MRI (lower left). Coronal T1-weighted MRI (lower right) shows clear wall enhancement (arrow). Modified from [36].

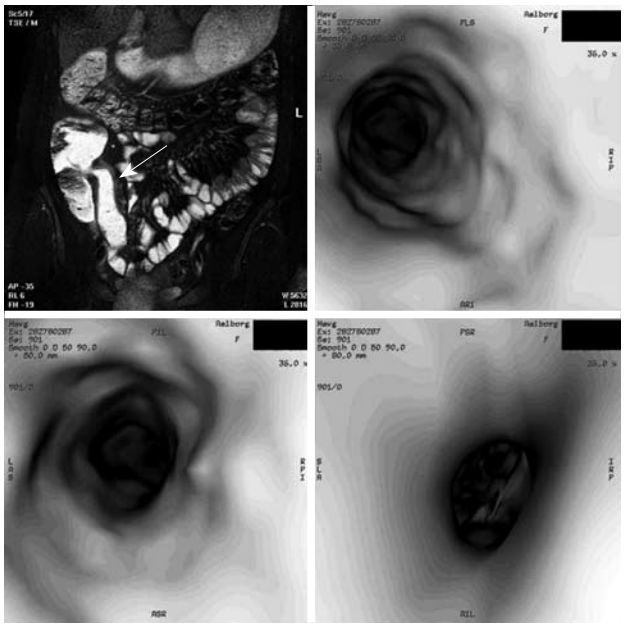

Figure 7 MRI of Crohn's disease. Virtual endoscopy views of the displayed diseased small bowel segment (arrow) shows mucosal nodularity, ileo-caecal narrowing and minor prestenotic dilatation. Modified from [36].

MRI is generally accepted as the gold standard examination in the staging of rectal cancers and inflammatory bowel diseases. Pelvic MRI, especially with endorectal coils, gives exact visualisation of infiltration of the rectal wall and perirectal fat allowing reliable TNM staging[34]. MRI of the small intestine has several advantages compared with conventional enteroclysis. It provides cross-sectional images without radiation hazards, and the entire small bowel can be visualised including other relevant abdominal pathology not directly related to the small bowel[26,35]. Luminal (stenosis, cobble stoning, and fissures), mural (wall thickening, and wall enhancement upon iv gadolinium) and exoenteric (mesenteric inflammation, fibrofatty proliferation, lymphadenopathy, hypervascularity, abscesses and fistulas) pathologies are visualised with high sensitivity and specificity (Figure 6)[35-38]. In particular, MRI is superior in the evaluation of fistulas in the anorectal region[39]. The optimal protocol for small intestinal MRI is not yet developed, since many different methods of preparation and imaging sequences exist. The intestine can be filled both orally or by intubation of the small intestine. Various positive and negative intestinal contrast materials exist. The use of water-based positive contrast agents are generally accepted with the addition of hyperosmotic substances (polyethylene glycol, methyl cellulose, bulk fibre laxative, mannitol, locust bean gum, *etc*) securing optimal distension of the entire small intestine[36,40-42]. Spasmolytics such as butylscopolamine and glucagon are usually administered to avoid motion-induced artefacts. The administration of intravenous contrast permits enhancement of hypervascular and hyperperfused areas allowing distinction between active and inactive inflammatory lesions. Acquisition of 3D images allows the possibility of virtual endoscopy which contributes to the detailed evaluation of a diseased bowel segment and the intraluminal display is easily recognized by gastroenterologists[36,43,44].

Our research group performed MRI and conventional enteroclysis in 36 patients with suspected Crohn's disease who underwent oral administration of plum juice and bulk fibre laxative[36]. Virtual endoscopy was performed with excellent demonstration of the mucosal surface (Figure 7). The main limitation of virtual endoscopy is the long and cumbersome computer post-processing and a high image quality is needed for this technique. However, this technique ensured sufficient distension of the small bowel for detecting small bowel changes. Pathological abdominal changes were found in 70% more patients using MRI compared with conventional enteroclysis[36]. MRI using this technique is preferable to conventional enteroclysis due to a superior demonstration of the entire small bowel pathology, low patient discomfort and absence of radiation exposure. In a study by Gourtsoyiannis *et al*[45], MR enteroclysis (MRE) was compared with conventional enteroclysis (CE) as the gold standard in 52 patients with small intestinal Crohn's disease. The sensitivity of MRE in the detection of superficial ulcers, fold distortion and fold thickening was 40%, 30% and 62.5%, respectively. The sensitivity of MRE in the detection of deep ulcers, cobble-stoning pattern, stenosis and prestenotic dilatation was 89.5%, 92.3% and 100%, respectively. Additional findings demonstrated on MRE images included fibrofatty proliferation in 15 cases and mesenteric lymphadenopathy in 19 cases. Hence, MRE strongly correlates with CE in the detection of individual lesions expressing small intestinal Crohn's disease, and provides

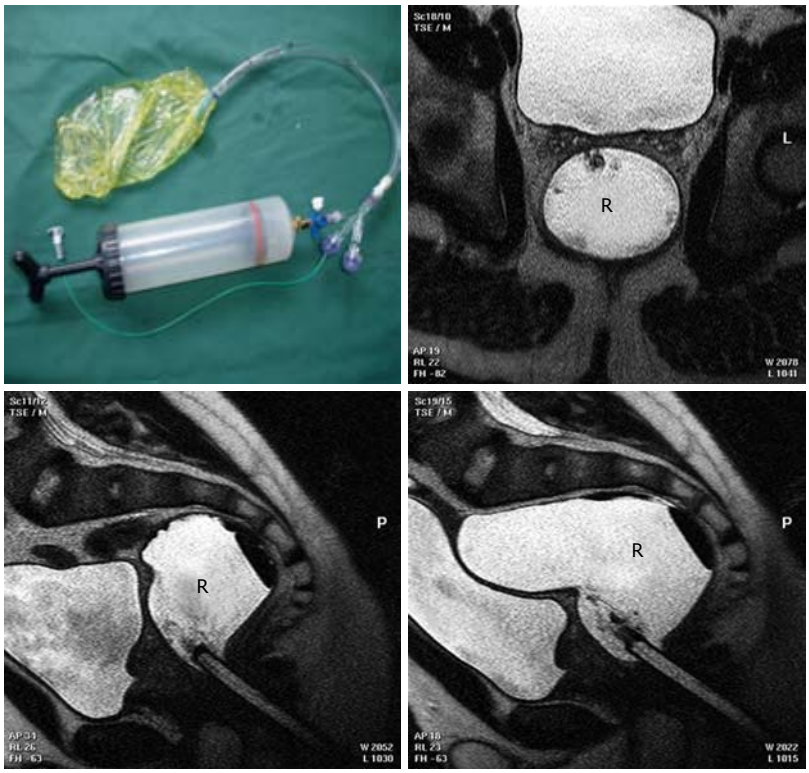

Figure 8 Stepwise distension of water filled balloon with simultaneous MRI and pressure recording. The rectal probe (upper left) allows rectal water distension and pressure measurement. MRI shows the distended water-filled bag in the rectum (R). The sagittal MRI (lower panel) shows the distension, elongation and relation to neighbouring structures at 100 mL and 300 mL inside the bag. Modified from [50].

additional information on the mesenteries. However, its capability in detecting subtle lesions is still inferior to CE. Additional functional cine-MRI will allow studies of intestinal motility with detection of intraabdominal adhesions due to surgery or inflammation[46]. The technique is also relevant for research studies on GI function and motility.

The technique of MRI colonography is also still developing[29,47,48]. Intestinal preparation is crucial in order to distinguish between polyps and intestinal residuals. Basically, the large intestine has to be cleaned and filled with water. New ways of faecal tracking with the oral application of negative contrast agents before the examination may allow less intestinal cleansing[49]. However, the method of MR colonography is not yet as sensitive as CT colonography in detecting smaller polyps.

Since MRI provides excellent soft tissue imaging capabilities without the use of radiation, it is ideally suited for research studies of the GI tract. Using advanced image processing, the three-dimensional geometry and mechano-sensory properties can be studied. Stepwise distension of water filled rectal and sigmoid balloons with simultaneous MRI and bag pressure recording was performed by our group (Figure 8)[50,51]. Based on the cross-sectional images, 3D models of curvatures, radii of curvature, tension and stress were generated and the circumferential and longitudinal strains were calculated (Figure 9). The distributions of the biomechanical parameters throughout the rectal and sigmoid surfaces were distinctly different between individuals and non-homogeneous throughout the colorectal wall due to its complex geometry. This complex geometry suggests that simple estimates of tension based on pressure and volume do not reflect the true 3D biomechanical properties of the intestine. This tool may in the future be useful in the research and clinical setting for assessing the geometry and mechano-sensory properties of visceral wall structures in health and disease.

PET

18F-fluorodeoxyglucose positron emission tomography (FDG-PET) has high accuracy in the detection and follow-up of oesophageal, colorectal and stomal cancers[52]. The advantage of PET is that metabolic changes often precede clear structural changes and, therefore, can be detected early in disease development. A combination of PET-CT is a powerful tool in the primary staging and assessment of any recurrent disease.

OTHER NOVEL TECHNIQUES

Other techniques such as impedance measurements and manometry which were initially not direct imaging modalities have developed more and more into techniques with imaging data display.

Impedance planimetry with assessment of multiple closely arranged cross-sectional areas can be displayed in 3D. This concept is known as the Functional Lumen Imaging Probe (FLIP) allowing direct on-line imaging of the luminal geometry of the GI tract[53,54]. This is particularly suitable for visualisation of the complex physiology of the GI sphincters, especially in the evaluation of gastro-oesophageal reflux and sphincter incompetence.

Oesophageal high-resolution manometry (HRM) with up to 36 pressure sensors allows on-line visual display with a spatio-temporal colour plot of oesophageal peristalsis[55]. The technique is explained

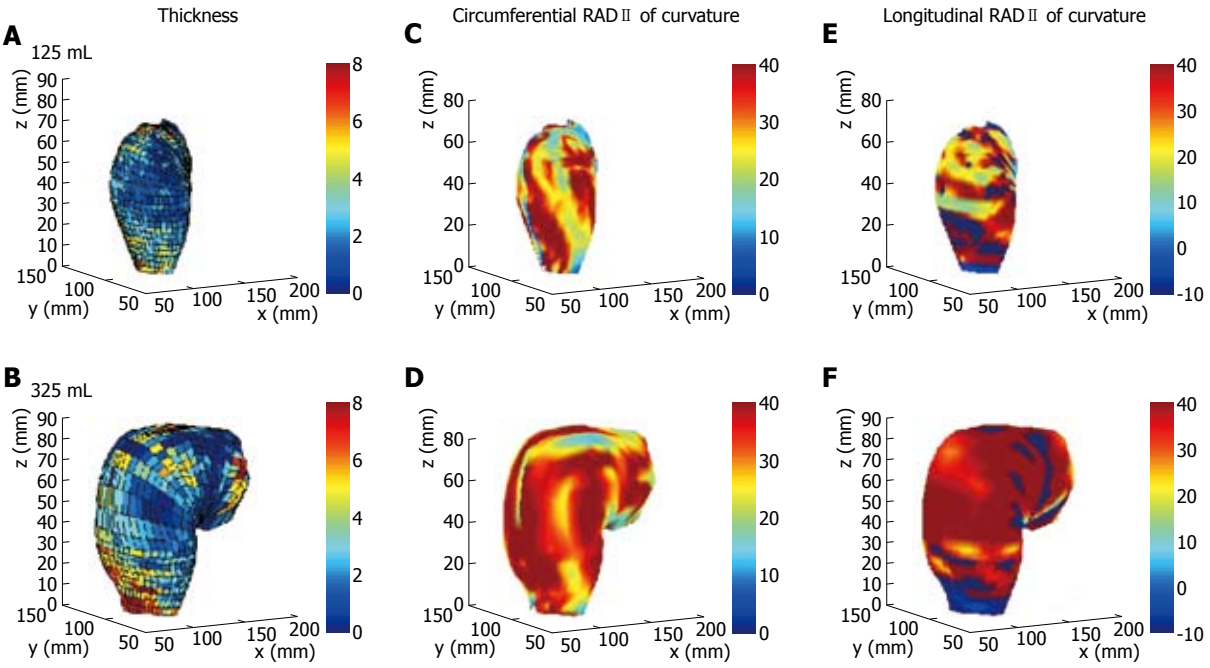

Figure 9 3D models of the rectum based on MRI and pressure recordings. The 3D distribution of the rectal wall thickness (A-B), circumferential (C-D) and longitudinal (E-F) principal radii of curvatures in one healthy volunteer at infused volumes of 125 mL (A, C, E) and 325 mL (B, D, F). The change in colour from blue to red during bag distension indicates an increase in rectal wall thickness or radius of curvature, i.e. increase in diameter. Modified from [50].

in detail in another paper in this issue. The recording reveals the complex motility of oesophageal bolus transport including sphincter function. Pathology related to oesophageal motor dysfunction is visualised with high accuracy[56]. Manometry can be combined with intraluminal impedance and pH measurements allowing further characterisation of reflux episodes (fluid *vs* air, acid *vs* non-acid).

Scintigraphy and single photon emission computed tomography (SPECT) are applied for emptying and motility studies of the GI tract[57,58]. Radionuclide transit/emptying scintigraphy is easy to perform, closely reflects physiology and provides quantitative data in the evaluation of several functional or motility disorders of the upper GI tract. However, scintigraphy has a low radiation burden. Like conventional radiography, dynamic scintigraphy with a radioactive liquid or semisolid bolus provides information on oesophageal motility useful in disorders such as nutcracker oesophagus, oesophageal spasm, non-cardiac chest pain, achalasia, oesophageal involvement in scleroderma, gastro-oesophageal reflux and monitoring response to therapy. Scintigraphy with a radiolabeled test meal represents the gold standard for evaluating gastric emptying in patients with dyspepsia, and evaluation of gastric function in various systemic diseases affecting gastric emptying. Similar scintigraphic methods are applied in the study of small intestinal and colonic transit. Recent radionuclide methods include dynamic antral scintigraphy and gastric SPECT for assessing gastric accommodation. However, US and MRI methods (see above) are still developing and the evaluation of functional GI diseases may in the future be subject to new and innovative techniques.

CONCLUSION

Imaging of the GI tract is essential in the diagnosis of GI diseases. This review highlights the capabilities of the newest techniques to explore the detailed morphology, biomechanical properties, function and pathology of the GI tract. The technological development is fast and the innovative potential enormous. Refinement of present modalities with faster image acquisition, higher resolution, increased computer power and improved software for post-processing are the main developing trends. Another trend is the development and refinement of "new sub-modalities" based on the traditional methods, and not least the fusion of different modalities into new multimodal concepts. Altogether, the future of GI imaging looks very promising, which will be of great benefit in clinical and research studies of GI diseases.

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Gastrointestinal tract modelling in health and disease

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Abstract

The gastrointestinal (GI) tract is the system of organs within multi-cellular animals that takes in food, digests it to extract energy and nutrients, and expels the remaining waste. The various patterns of GI tract function are generated by the integrated behaviour of multiple tissues and cell types. A thorough study of the GI tract requires understanding of the interactions between cells, tissues and gastrointestinal organs in health and disease. This depends on knowledge, not only of numerous cellular ionic current mechanisms and signal transduction pathways, but also of large scale GI tissue structures and the special distribution of the nervous network. A unique way of coping with this explosion in complexity is mathematical and computational modelling; providing a computational framework for the multilevel modelling and simulation of the human gastrointestinal anatomy and physiology. The aim of this review is to describe the current status of biomechanical modelling work of the GI tract in humans and animals, which can be further used to integrate the physiological, anatomical and medical knowledge of the GI system. Such modelling will aid research and ensure that medical professionals benefit, through the provision of relevant and precise information about the patient's condition and GI remodelling in animal disease models. It will also improve the accuracy and efficiency of medical procedures, which could result in reduced cost for diagnosis and treatment.

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Key words: Gastrointestinal tract; Computational modelling; Biomechanics; Remodelling; Disease

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INTRODUCTION

The gastrointestinal (GI) tract, also called the digestive tract or the alimentary canal, is the system of organs within multicellular animals that takes in food, digests it to extract energy and nutrients, and expels the remaining waste. The major functions of the GI tract are digestion facilitated by motility, secretion and absorption. The various patterns of GI tract function are generated by the integrated behaviour of multiple tissues and cell types. Medical imaging methods such as ultrasonography[1,2], magnetic resonance imaging (MRI)[3,4], and endoscopic ultrasound (EUS)[5,6] are well known stand-alone clinical methods that can disclose structural and functional abnormalities of the GI tract. However, a thorough study of the GI tract requires understanding of the interactions between cells, tissues and gastrointestinal organs in health and disease. This depends on knowledge, not only of numerous cellular ionic current mechanisms and signal transduction pathways, but also of large-scale GI tissue structures and the special distribution of the nervous network. A unique way of coping with this explosion in complexity is mathematical and computational modelling; providing a computational framework and Information Communication Technology (ICT) based tools for multilevel modelling and simulation of the human gastrointestinal anatomy and physiology[7-9]. Computer-based analysis, visualisation, modelling and simulation are used routinely in fields such as engineering, meteorology or traffic control to understand the behaviour and outcomes of new designs and impact of

external phenomena well in advance of their realisation, thereby avoiding costly failures. In GI tract studies, this approach is not common, mainly because we still lack those models that can emulate the behaviour of the human body. Nevertheless, exploration of the GI tract has dramatically improved by the introduction of cross sectional imaging modalities such as Computed Tomography (CT) and Magnetic Resonance Imaging (MRI), which have revolutionised the way in which many conditions are diagnosed and treated. The ability to examine, in detail, structures inside the GI tract without resorting to surgery, has allowed clinicians to diagnose problems and plan corrective procedures with a minimum of risk to the patient[10-11]. In order to continue this exploration, it will be necessary to complement the traditional approach with an integrative approach that combines observation, theory and prediction across the temporal and dimensional scales, across scientific disciplines, and across the anatomical subsystems, all of which reflect the rather artificial divisions described.

The aim of this review is to describe the currently status of biomechanical modelling work on the GI tract in humans and animals that can be further used to integrate the physiological, anatomical and medical knowledge of the GI system.

ANATOMY AND FUNCTION OF THE GI TRACT

The GI tract is a continuous channel through the body with the biliary and pancreatic ducts as major side-branches. The GI tract consists of a series of organs, which resemble one another in constitution, being variously arranged as cylinders, spheroids, or intermediate forms. The main functions of the GI tract are transport and digestion of food. The different segments show a large variation in morphology and muscle mechanical properties, i.e. the oesophagus mainly serves to quickly transport the food bolus from the mouth to the stomach where the food in the stomach is stored for some time whilst simultaneously being broken down into smaller components. The GI sphincters serve to separate the GI tract into compartments. However, the gut is also important for immune functions[12]. The wall of the GI tract is typically composed of four layers, i.e., the mucosa, submucosa, muscle and serosa (some parts are called the adventitia where there is no epithelium) (Figure 1). The muscle layer consists of an outer longitudinal and an inner circular muscle layer. The collagen-rich submucosa and mucosa layers are inside the muscle layer. Another thin layer of muscle, the muscularis mucosae, exists almost throughout the entire tract. The motions of the GI tract accomplish a net antegrade flow in order to mix the contents and move them across the surface where absorption occurs. The contractile patterns and transit vary greatly from one part of the tract to another. The GI wall movements during digestion and absorption are the consequence of contractions of the two layers of smooth musculature. Contractions of the longitudinal muscle layer shorten the gut wall, whereas the peristaltic contractions of the circular muscle layer, in contrast, mainly produce forward transit with relatively little mixing. The contractions of circumferential and longitudinal muscles occur together, most of the time. The enteric nervous system (ENS), composed of both the myenteric (inter-muscular) plexus and the submucosal plexus, is distributed in the GI tract from the oesophagus to the internal anal sphincter[13]. A network of nerves of the myenteric plexus is embedded in the loose collagen matrix between the longitudinal and circular muscle layers (Figure 1). This set of nerves is essential for the regulation of the contractions of the adjacent musculature. Between the nerve endings and smooth muscle are the interstitial cells of Cajal (ICCs), which have been shown to be critical for the generation and propagation of the electrical slow waves that regulate the phasic contractile activity of GI smooth muscle, and for mediating neurotransmission from enteric motor neurons to smooth muscle cells[14,15]. The ENS ensures that the GI tract can fulfil essential tasks even when isolated from the rest of the body. The GI tract is - on the other hand - unable to work normally without the integrative functions of the ENS. Malfunctions of the ENS are increasingly recognized as underlying factors in many GI diseases. The exogenous nerves running together with the sympathetic and parasympathetic nervous systems are also important in regulating blood flow and secretion *etc*[16]. They also encode the conscious sensations from the gut such as fullness, urge to defecate and pain[17]. Medical imaging methods such as ultrasonography, MRI, and endoscopic ultrasound (EUS) are well known stand-alone clinical methods that can disclose structural and functional abnormalities of the GI tract[6,18]. Therefore, modelling analysis based on the anatomy and structure of the GI tract and different imaging methods can be applied to the problems related to function and pathophysiology.

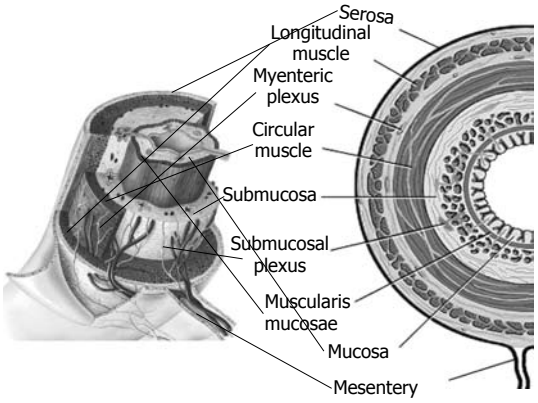

Figure 1 Schematic diagram of the GI tract.

DISEASE CAUSING TISSUE AND STRUCTURE REMODELLING OF THE GI TRACT

The GI tract, like other hollow organs such as the

Table 1 Diseases causing histomorphological and biomechanical remodeling of GI tract[19-45]

Diseases	Species	Test organs	Histomorphometric remodeling			Biomechanical remodeling		
			WT	WA	LT	OA	RES	Stiffness
Type Ⅰ diabetes	Human	Esophagus	↑	ND	ND	ND	ND	Circ NC Long↑
		Duodenum		ND	ND	ND	ND	Circ NC Long↑
	Rat	Esophagus	↑	↑	Mu↑Su↑Ms↑	↓	↓	Circ↑Long ND
		Duodenum	↑	↑	Mu↑Su↑Ms↑	↓	↓	Circ↑Long↑
		Jejunum	↑	↑	Mu↑Su↑Ms↑	↑	↑	Circ↑Long↑
		Ileum	↑	↑	Mu↑Su↑Ms↑	↑	↑	Circ↑Long↑
		Colon	↑	↑	Mu↑Su↑Ms↑			Circ↑Long↑
Type Ⅱ diabetes	Rat	Esophagus	↑	↑	Mu↑Su↑Ms↑	↓	↓	Circ↑Long ND
		Stomach	ND	ND	ND	ND	ND	Circ↑
Systemic sclerosis	Human	Duodenum	ND	ND	ND	ND	ND	Circ↑
Ulcerative colitis	Mice	Colon	↑	↑	Mu↑Su↑Ms↑	↑	↑	Circ↑Long↑↑
Fasting	Rat	Duodenum	↓	↓	Mu↓ Su↓	↑	↑	Circ↓ Long↓
		Jejunum	↓	↓	Mu↓ Su↓	↑	↑	Circ↓ Long↓
		Ileum	↓	↓	Mu↓ Su↓	↑	↑	Circ↓ Long↓
Low protein diet	Mink	Duodenum	↓	↓	NC	NC	NC	Circ↓ Long NC
		Jejunum	↓	↓	Mu↓ Su↓	↓	↓	Circ↓ Long NC
		Ileum	↓	↓	Mu↓ Su↓ Ms↓		↓	Circ↓ Long NC
Partial obstruction	Opossum	esophagus	ND	ND	ND	ND	ND	Circ↑Long ND
	Guinea pig	Jejunum	↑	↑	Mu↑Su↑Ms↑	↓	↓	Circ↑Long ND
Osteogenesis imperfecta	Mice	Esophagus	↓	↓	Mu↓ Su↓ Ms↓	↑	↑	ND
Irradiation	Mice	Rectum	↑	↑	ND	↑	↑	ND
Small bowel resection	Rat	Jejunum	↑	↑	Mu↑Su↑Ms↑	↑	↑	NC
		Ileum	↑	↑	Mu↑Su↑Ms↑	↑	↑	NC
EGF treatment	Rat	Esophagus	↑	↑	Mu↑Su↑Ms↑	↑	↑	Circ↑Long ND
		Duodenum	↑	↑	Mu↑Su↑Ms↑	↑	↑	Circ↑Long NC
		Jejunum	↑	↑	Mu↑Su↑Ms↑	↑	↑	Circ↓ Long↑
		Ileum	↑	↑	Mu↑Su↑Ms↑	↑	↑	Circ↓ Long↑
		Colon	↑	↑	Mu↑Su↑Ms↑	↑	↑	ND

WT: Wall thickness; WA: Wall cross-sectional area; OA: Opening angle; RES: Residual strain; LT: Layered wall thickness; Mu: Mucosa; Su: Submucosa; Ms: Muscle; Circ: Circumferential direction; Long: Longitudinal direction; ND: Not done; NC: No change.

heart, blood vessels, urinary bladder and the urethra, is functionally subjected to dimensional changes. Hence, biomechanical properties are of particular functional importance. Data on the biomechanical properties are crucial for the understanding of the normal function of the GI tract and dysfunction due to disease because, (1) peristaltic motion that propels the food through the GI tract is a result of interaction of the passive and active tissue forces and the hydrodynamic forces in the food bolus and (2) remodelling of the mechanical properties reflects the changes in the tissue structure that determine a specific sensory-motor dysfunction.

Human studies have documented that diabetes melitus[19] and systemic sclerosis[20] induce biomechanical GI remodelling. Using different animal models (Figure 2), we have demonstrated that biomechanical and histomorphological remodelling occur in the GI tract due to normal physiological growth[21,22], malnutrition[23,24], inflammation, obstruction[25-27], bowel resection[28], diabetes[29-33], radiation injury[34], collagen changes[35,36] and EGF treatment. The morphometric properties are best described at the zero-stress state where no internal or external forces deform the tissue. Furthermore, knowing the zero-stress configuration is essential in any mechanical analysis since it serves as the reference state for computing stress and strain under physiological or pathophysiological conditions. With reference to the zero-stress state, combining the morphometry data and pressure data, we can compute the stress-strain relationship of the GI wall. The stress-strain distribution mainly reflects the elastic properties of the GI tract. Changes in the elastic properties reflect structural remodelling of the GI wall in different diseases. Therefore, we consider the opening angle of the zero-stress state, residual strain and stress-strain relationship as the most relevant biomechanical parameters to describe diseases causing GI remodelling. Generally, diseases and factors inducing tissue overgrowth, such as diabetes[37-40], obstruction and EGF[41-45] treatment, increase GI wall stiffness; whereas factors reducing tissue growth, such as fasting and low protein diet decrease GI wall stiffness. The more collagen in the GI wall, the stiffer the wall is[20], and vice versa. However, the effect of different factors on the opening angle and residual strain of GI tract depend on the changes of layered structure. Fung's hypothesis of non-uniform remodelling states that if the inner wall grows more than the outer wall, the opening angle will increase[46] whereas if the outer wall grows more than the inner wall, the opening angle will decrease. Table 1 summarizes the histomorphological and biomechanical remodelling of the GI tract caused by different diseases.

It is well known that mechanosensation is important for GI function. Mechanosensitive nerve endings exist extensively in the GI tract where they serve a critical role in homeostasis. The mechanosensitive afferents in

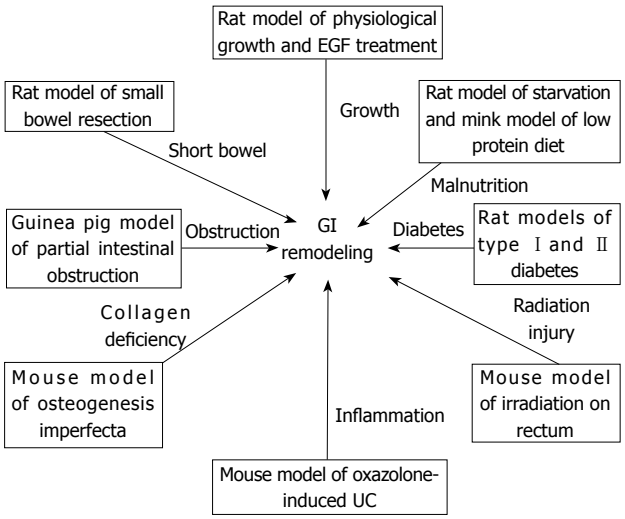

Figure 2 Disease-induced GI remodeling in animal models.

the intrinsic and extrinsic pathways have been described as low-, wide-dynamic- or high-threshold tension-receptors[12]. Therefore, the GI tract structure, as well as the stress and strain distribution in the wall, is important for the GI sensory and motor function. The GI wall structure or deformation changes caused by a disease will alter the relative positions of the mechanosensitive afferents (zero setting of the mechanosensitive afferents). The biomechanical remodelling by the disease, such as alterations of residual strain, stress distribution and wall stiffness, will alter the tension and stress distribution of the mechanosensitive afferents. As a result, the perception and motility of the GI tract will also change. Therefore, the morphological changes and biomechanical remodelling of the GI tract are likely to affect the function of mechanosensitive afferents in the GI wall and further affect the motor and sensory functions.

BIOMECHANICAL MODELLING OF THE GI TRACT

The use of numerical models and, in particular, of finite element models has been extensively studied in the field of soft tissue mechanics because of the potential they offer in the analysis of the mechanical behaviour of morphologically complex structures, with high structural hierarchy and constituents with non-linear behaviour[47,48]. The effectiveness of numerical models depends on reliable reconstructions of the morphometry of the anatomical site under investigation, the specific loading and boundary conditions, as well as the definition of constitutive models capable of describing the mechanical response of the single tissues. Gastroenterology research has traditionally been based on experimental approaches rather than on mathematical modelling. Most of the previous modelling efforts in the biological area were in the cardiac and lung field; but, other areas are in now being developed. However, in the past five to ten years several groups have independently started to model the GI tract.

The large morphological complexity of the GI tract and the variability in the different parts of the tract are well known. The complexity increases in the characteristic folds of the connection regions[49-51]. With regard to the structural conformation of GI tract tissues, the inner mucosa and submucosa layers are surrounded by the outer muscular and serosa layers. Collagen fibres of submucosa form a complex network and are oriented in different directions. The muscular layers have muscle fibres oriented in the circumferential direction (circumferential muscle layer) or the longitudinal direction (longitudinal muscle layer). As a consequence, the GI tract tissue must be considered as a multi-layered composite material, made up of tissues with different mechanical characteristics[12,52-54]. Some of the components show a specific spatial disposition of the sub-structures, such as collagen and muscle fibres, and are studied by means of a constitutive formulation already adopted in other fields for the mechanics of biological tissues, based on the theory of fibre-reinforced materials[55-57]. The most current investigations of the GI tissue properties are mainly focused on seeking the constitutive equation and the associated constitutive parameters of the physiological or pathological status. To date, most GI structure and tissue property studies have been based on animal experiments. Medical device development has made it possible to study the mechanical behaviour of the GI tract *in vivo*[58-62].

The methods and current developments in studies of the biomechanical properties of normal and disease remodelled GI tissues have been described in the above sections. Hereafter, the establishment of morphometric-related modelling of the GI tract will be briefly introduced. According to the reconstruction methods on GI modelling, the establishment of the GI models can be divided into *in vivo* medical images-based models, the anatomical-based models and the theoretical analysis-based models.

In vivo medical images-based GI models

Advances in imaging are introduced initially as research tools and subsequently as clinical diagnostic tests. Medical image-based 3-D models of *in vivo* GI organs have characterized the oesophagus, stomach, small intestine, sigmoid colon, oesophageal gastric junction and the rectum, based on cross-sectional imaging using ultrasonography, computed tomography (CT), Functional Luminal Imaging Probe (FLIP) or magnetic resonance imaging (MRI)[3,4,63-68]. With the development of the medical devices such as impedance planimetry, it is now possible to record the mechanical parameters such as the luminal pressure simultaneously with the cross-sectional medical images. Therefore, the *in vivo* mechanical behaviour of the organs can be computed on the basis of the reconstructed GI morphometric models and the recorded mechanical parameter. A reconstructed sigmoid-colon model and the corresponding tension and stress distribution on the model are illustrated as an example in Figure 3. Detailed descriptions of *in vivo* GI

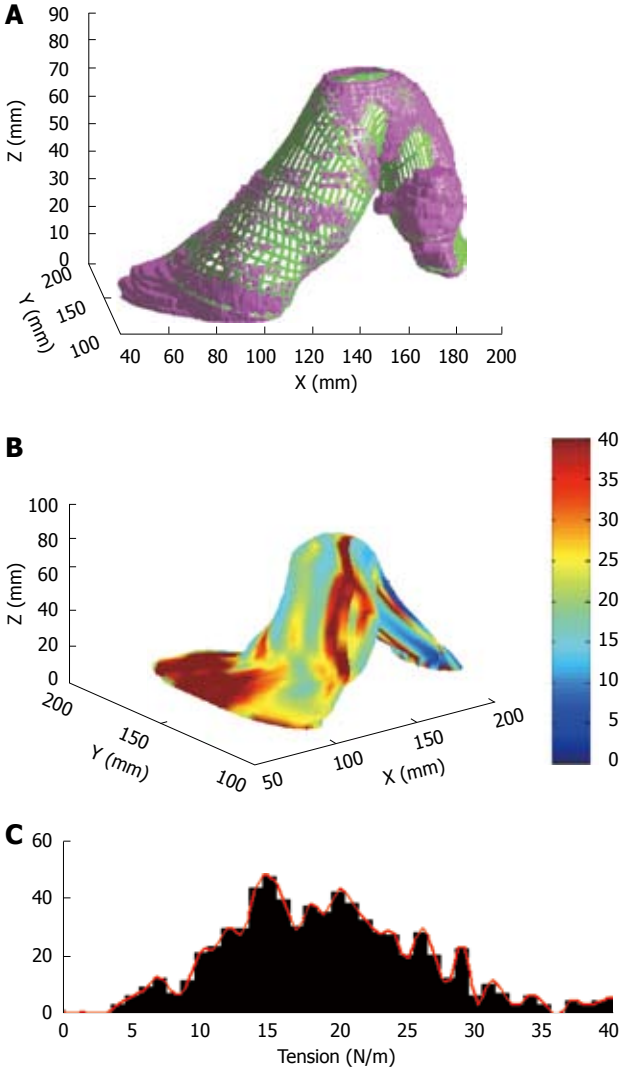

Figure 3 A reconstructed sigmoid-colon model and the corresponding tension distribution. A: A representative sigmoid colon model with the distension volume of 200 mL. The model with purple colour is the solid model generated directly from the MR images, and the green mesh is the smoothened surface, the comparison between the solid model and smoothened surface indicates that the smoothened model fits well with the original solid model; B: The circumferential curvature distribution on the surface models; C: Tension distribution of the sigmoid colon surface model.

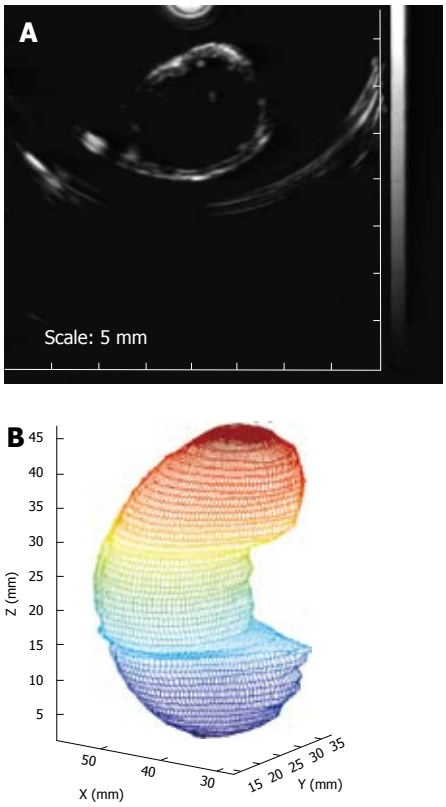

Figure 4 An example of the anatomically based *in vitro* rat stomach model generated from ultrasonic scanning. A: A representative CT scanning of a cross sectional slice of an *in vitro* rat stomach; B: The reconstructed gastric model on the basis of the CT scanning on the *in vitro* rat stomach. The distance between cross sectional slices was 1 mm, the colour change from blue to red means the increase of the stomach length in z direction.

modelling analysis can be found in studies of Liao *et al*[66] and Frokjaer *et al*[3,4].

Anatomy-based GI modelling

For modelling analysis using the *in vivo* image based models, only the tension or stress was computed on the basis of three-dimensional surface geometry using the Laplace's equation, and the wall thickness. The tissue structure was, therefore, not taken into account. To aid in understanding of the relationship between the structure and function of the GI tract in healthy and diseased states, an anatomically-based finite element model is needed. The anatomically based visualization GI model is now commercially available, however the GI anatomy based numerical modelling analyses are mainly been done by Andrew Pullan's group so far, and all models were built from the Visible Human Project[69-71]. The Visible Human project provided a set of cross sectional images of a human cadaver. On each image, data points around the organ boundary of interest were created and then the geometry models were constructed on the basis of the distinguished data clouds[71-75]. The anatomically-based models have now been used to investigate normal and pathological electrical activity of the stomach and small intestine[71-74], the muscle functions on the gastro-oesophageal junction during swallowing[75] and the blood flow in the mesenteric arterial system of the human intestine[69,70]. An example of the anatomically-based *in vitro* rat stomach model generated from ultrasonic scanning is illustrated in Figure 4.

Theoretical analysis-based GI modelling

The morphological complexity of the GI organs makes it difficult to build the anatomically-based finite element models. Hence, some numerical models of the GI organs were built on the basis of theoretical analysis by simplifying the complex GI morphometry as a regular geometry such as a circular cylinder for the oesophagus[52,57,64,68,76] and a sphere for the stomach pouch[77] and some regular tubes for describing the antrodudenal junction[78] and the oesophago-gastric junction[79]. In the morphometrically-simplified model, most of the biomechanical features such as the tissue structure, tissue properties and bolus flow can be

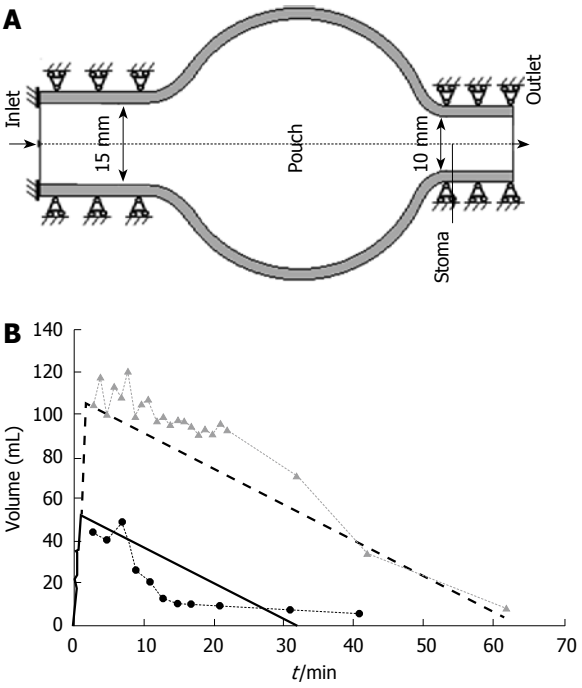

Figure 5 A simplified pouch model for describing the gastric emptying of a patient treated for obesity. A: A representative pouch model of midsized pouch with stoma diameter of 10 mm, B: Volume history in the filling and emptying phases in the mid-sized and large pouch models with stoma diameter of 10 mm. The solid line represents the mid-sized pouch, and the dotted line the large pouch. Circles and triangles represent volume data of the recorded clinical emptying curve for the mid-sized and large pouch. Pouch and stoma are a small fundic cavity and a corresponding narrow outlet between pouch and the rest of the stomach in gastroplasty and gastric bypass procedures for obesity.

expressed mathematically and, thus, the mechanical function of the GI tract can be simulated. The simplified GI tract models have existed for describing the muscle function[53,68,76], food transportation[77,78,80,81] and blood flow[65] in the GI tract in healthy and diseased situations. A simplified pouch model for describing the gastric emptying of a patient treated for obesity is illustrated in Figure 5 as an example. As can be seen, the emptying curves for the pouch based on the simplified model agree well with the clinical results.

PERSPECTIVE

GI modelling studies are focused on patient-specific computational modelling and simulation for prediction of disease or early diagnosis by integrating patient specific knowledge and predispositions obtained in biomedical imaging. Modelling studies of the GI tract will advance our understanding of the mechanisms of GI function and diseases, such as dyspepsia and visceral pain. Furthermore, an integrated GI tract simulation model will be beneficial for medical education, and for evaluation of the efficacy and safety of new drugs. The challenge of GI modelling is to develop mathematical and computational models of structure-function relations appropriate to each (limited) spatial and temporal domain, and then to link the parameters of a model at one scale to a more detailed description of structure and function on the adjacent levels. The present analytical tools can thus be integrated in order to analyze complex structures for understanding biomechanical behaviour in other visceral organs and be further integrated as a global GI model. The mechanical behaviour-related aspects of diseases of the sigmoid colon (diverticular disease, irritable bowel syndrome, *etc*), small bowel (motility disorders), stomach (motility disorders, non-ulcer dyspepsia, *etc*) and oesophagus (oesophagitis, gastro-oesophageal reflux disease, non-cardiac chest pain, *etc*) can be further developed by applying modified versions of the present models.

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Translational pain research: Evaluating analgesic effect in experimental visceral pain models

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Abstract

Deep visceral pain is frequent and presents major challenges in pain management, since its pathophysiology is still poorly understood. One way to optimize treatment of visceral pain is to improve knowledge of the mechanisms behind the pain and the mode of action of analgesic substances. This can be achieved through standardized experimental human pain models. Experimental pain models in healthy volunteers are advantageous for evaluation of analgesic action, as this is often difficult to assess in the clinic because of confounding factors such as sedation, nausea and general malaise. These pain models facilitate minimizing the gap between knowledge gained in animal and human clinical studies. Combining experimental pain studies and pharmacokinetic studies can improve understanding of the pharmacokinetic-pharmacodynamic relationship of analgesics and, thus, provide valuable insight into optimal clinical treatment of visceral pain. To improve treatment of visceral pain, it is important to study the underlying mechanisms of pain and the action of analgesics used for its treatment. An experimental pain model activates different modalities and can be used to investigate the mechanism of action of different analgesics in detail. In combination with pharmacokinetic studies and objective assessment such as electroencephalography, new information regarding a given drug substance and its effects can be obtained. Results from experimental human visceral pain research can bridge the gap in knowledge between animal studies and clinical condition in patients suffering from visceral pain, and thus constitute the missing link in translational pain research.

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Key words: Visceral pain; Analgesics; Pharmacokinetics; Pharmacodynamics

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INTRODUCTION

Deep muscular and visceral pain is very frequent and causes major challenges in pain management[1]. Visceral pain is different from somatic pain because it is more diffuse, hard to localize, accompanied by autonomic reflexes, and very often described with an associated somatic referred pain. Pathophysiology and the mechanisms behind visceral pain conditions remain poorly understood. This lack of knowledge makes treatment of visceral pain challenging and often suboptimal.

Non-opioids are often insufficient in relieving pain to an acceptable level in patients suffering from severe pain originating from the gastrointestinal tract[2]. On the other hand, treatment with traditional μ-opioid agonists may not be optimal. It often fails to relieve pain sufficiently, and at the same time causes side effects such as constipation, euphoria, sedation and nausea. These adverse effects are mainly mediated through μ-receptors in the central nervous system (CNS). To encompass these problems, new therapeutic approaches have addressed opioids interacting with the peripheral κ-receptor, NMDA-antagonists and adjuvant analgesics (antidepres-

sants and anticonvulsants)[3-7].

In experimental pain models, it is important to have a robust pain measure to obtain a reliable model and to detect the analgesic effect[8]. In standardized experimental human pain models, the investigator can control the induced pain (including modality, localization, intensity, frequency and duration), and provide quantitative measures of the responses. Hence, confounding factors such as sedation, nausea and general malaise that underlie clinical pain can, to a large extent, be controlled or avoided[9]. Different experimental stimulations can be used to induce visceral pain. Thermal, mechanical, electrical and chemical stimulations can be performed with a multi-modal approach, for which, different receptor types, pathways and mechanisms can be activated. This may act as a proxy for some of the mechanisms involved in clinical visceral pain conditions[10]. Such a multi-modal stimulus regimen can be used in conjunction with a multi-tissue approach in which skin, muscles and viscera are stimulated[9].

Pharmacokinetic and pharmacodynamic (PK-PD) modeling of analgesics can be used to identify and explain potential differences between the analgesic substances in their mechanism of alleviating pain. The effect depends on the concentration at the site of action. The site of action (biophase) for many analgesic substances is within the CNS, while most pharmacokinetic studies measure analgesic concentrations in plasma. When PK-PD modeling is performed, it is, therefore, important to account for a possible delay between plasma concentration and effect[11].

PK-PD MODELING

There are various approaches to the study of opioid pharmacokinetics and pharmacodynamics (Figure 1). These can be classified according to their relative advantages and disadvantages.

Animal studies

These studies allow the investigation of fundamental mechanisms (such as cerebral equilibration rates) and the collection of arterial and venous blood concentration data. However, the dynamic information [e.g. tail flick times, changes in electroencephalography (EEG) or magnetic resonance imaging (MRI) signals] cannot be readily related to analgesia in humans. Representative studies include sheep studies performed at the University of Adelaide, Australia where a model to study the relationship between plasma and CNS concentration has been developed by Upton *et al*[12,13]. They have previously used a sheep preparation to examine the cerebral kinetics and dynamics of analgesic drugs used in the perioperative period. Physiological PK-PD models developed in sheep have been adapted to assess the clinical profile of these drugs in humans[14]. One sheep study has shown that the faster analgesic onset with oxycodone compared to morphine might be explained by a faster equilibration between blood and brain for oxycodone[15].

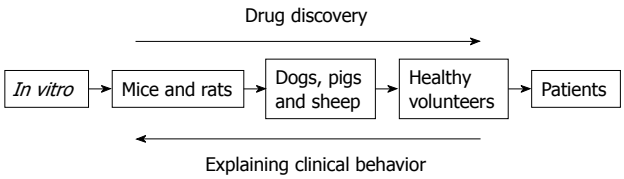

Figure 1 Understanding the effect of analgesics. An overview of the different levels of investigation of analgesic effects. A good deal of attention has been focused on progressing from left to right in drug discovery. Less attention has been focused on progressing from right to left. Reproduced with permission from Upton *et al*[33].

Surgical patient studies

These studies are typically conducted in patients just before or during surgery when patients have an arterial cannula (often for patient management). As the patients are generally sedated (and cannot report pain) and the dose is high, the pharmacodynamic information is generally derived from changes in EEG. A representative study is that of Poyhia *et al*[16] in which EEG was used to quantify the CNS effects of oxycodone during anesthesia for primary coronary artery bypass grafting.

Volunteer and awake patient studies

For ethical reasons, these subjects usually only have a venous cannula placed in an arm vein. However, they can report highly relevant dynamic information such as pain and sedation scores, and can be studied using doses and routes that are directly relevant to clinical practice. Representative studies are the opioid study by Staahl *et al*[17], or the intranasal fentanyl studies of Christrup *et al*[18] and Foster *et al*[19]. Differences in the site of action for opioids may be reflected in the delay between opioid blood and CNS concentration and the analgesic effect. These differences might be more pronounced in diseases in which liver and kidney function are reduced or affected. Understanding these differences has implications for interpretation of PK-PD opioid studies, and provides insight into optimal clinical analgesic management of visceral pain[17]. A robust pain assessment is needed to obtain a reliable model of the PK-PD relationships for opioids. Experimental pain models in healthy volunteers provide less variable and less confounded pain measures, which are suitable for PK-PD modeling. A neurophysiological objective assessment of pain response and analgesic effect is EEG, which also can support the subjective findings in experimental pain studies. Previous investigations have shown that quantitative (spectral) analysis of the increase in delta frequency band of the resting EEG is a suitable biomarker for the PK-PD correlation of opioids[20,21]. In a study of biophase kinetics within the PK-PD analysis of a wide range of opioids, morphine showed profound hysteresis between the blood pharmacokinetics and EEG effect[22]. Groenendaal *et al*[22] have concluded that within the wide range of opioids used in their study, only morphine displayed complex biophase distribution kinetics, which can be explained by its relatively low permeability of the blood-brain barrier and its interaction with active transporters present at the barrier.

TESTING OF ANALGESICS IN EXPERIMENTAL VISCERAL PAIN MODELS

Until now, only a few studies investigating the effect of analgesics in visceral experimental pain in healthy volunteers have been performed. Only two of these studies assessed the PK-PD relationship[17,23].

Opioids

Morphine: Morphine is a highly potent opiate analgesic drug and is the principal active agent in opium, and is the prototypical opioid. Morphine is one of the few opioids to have been evaluated in human experimental visceral pain models[24]. Comparing somatic and visceral tissue, differences in opioid analgesia have been observed. Morphine does not affect somatic pain. Morphine analgesia is significantly better than placebo in attenuating mechanical and electrical esophageal pain, but not thermal esophageal pain.

Oxycodone: Oxycodone is a semi-synthetic opioid with analgesic effect and, as with morphine, has been evaluated in human experimental visceral pain models[24]. As for morphine, tissue differences in opioid analgesia have been observed. One study has shown that oxycodone is significantly better than placebo in attenuating mechanical, electrical and thermal esophageal pain. Furthermore, oxycodone has a superior effect on visceral pain compared to morphine[24]. This indicates that oxycodone may interact with other visceral opioid receptors more than morphine does. This reflects the clinical situation in which visceral pain, in contrast to somatic pain, can be difficult to treat with traditional μ-opioid agonists[25].

Lalovic *et al*[23] have studied the pharmacokinetics and pharmacodynamics of oxycodone in healthy human volunteers; measurements included the time course of plasma concentrations and urinary excretion of metabolites, along with the time course of miosis, and subjective opioid side effects. The contribution of circulating metabolites to oxycodone pharmacodynamics has been analyzed by PK-PD modeling. The human study was complemented by *in vitro* measurements of opioid receptor binding and activation studies, as well as *in vivo* studies of the brain distribution of oxycodone and its metabolites in rats. Noroxycodone and noroxymorphone are the major metabolites in the circulation with elimination half-lives longer than that of oxycodone; but, their uptake into the rat brain is significantly lower compared with that of the parent drug. PK-PD modeling has indicated that the time course of pupil constriction in healthy volunteers is fully explained by the plasma concentration of the parent drug oxycodone. The metabolites do not contribute to the central effects, either because of their low potency or low abundance in circulation, or as a result of their poor uptake into the brain[23].

A pronounced delay between the plasma concentrations and analgesia will produce hysteresis when the analgesic effect is plotted against plasma concentration. This is characteristic for opioids and has been shown previously to be caused partially by the rate of transport of the opioid into the CNS (across the blood-brain barrier)[26], but also by receptor-mediated cascades[13,27]. Traditionally, hysteresis is collapsed by the implementation of a theoretical effect compartment between the plasma compartment and effect (i.e. the effect is not delayed compared to the drug concentration in this compartment)[11].

In visceral pain assessments in healthy volunteers, obvious differences are seen between oxycodone and morphine PK-PD profiles. The effect of morphine is generally described through an effect compartment, whereas oxycodone tends to be more directly linked to the plasma concentration, because no hysteresis is produced[17]. This supports the results of Lalovic *et al*[23] and the theory that oxycodone acts partially at a peripherally located receptor. One hypothesis is that this peripherally located receptor is the κ receptor, since there is some evidence that oxycodone has a partial effect at the κ opioid receptor[24,28]. In contrast to other opioid receptor types, for which central effects dominate, the peripheral κ receptor may also be important for visceral analgesia[3,29,30]. Staahl *et al*[17] have confirmed that morphine and oxycodone have somewhat different PK-PD relationships in attenuation of visceral pain and, therefore, most likely act at receptors situated in different physiological compartments. These results partly address the question: to what extent do the cerebral pharmacokinetics of a drug contribute to its clinical behavior? Furthermore, they provide insight into optimal clinical analgesic management of visceral pain.

N-methyl-D-aspartic acid (NMDA)-antagonists

Ketamine is classified as an NMDA receptor antagonist. Ketamine has also been found to bind to opioid receptors. It has the added benefit of counteracting spinal sensitization or wind-up phenomena experienced with chronic pain. It is primarily used for the induction and maintenance of general anesthesia, usually in combination with some sedative drug, because otherwise unwanted psychological side effects can occur.

The analgesic effect of ketamine has been investigated in several experimental pain models. During visceral distension, pain and unpleasantness are decreased by ketamine[4]. Apparently, deep muscular or visceral pain is treated more successfully than superficial pain[4,31]. This supports the findings in other human studies in which deep pain activates central mechanisms (involving the NMDA receptor) such as summation more quickly than superficial pain does[32].

Hyperalgesia to electrical pain has been induced in the esophagus by acid infusion. It has been shown that ketamine prevents development of hyperalgesia and reverses induced hyperalgesia[5].

Antidepressants

Imipramine is a tricyclic antidepressant of the dibenzazepine group. Imipramine is similar in structure to some muscle relaxants, and has a significant analgesic effect, and, therefore, is very useful in some pain conditions. As an example of experimental pain testing of the effect of imipramine, non-nociceptive sensation and pain to

distension of the esophagus have been investigated by Peghini *et al*[6]. In this study, only stimulation within the painful range was affected, which shows a pain-specific action of imipramine.

Amitriptyline is a tricyclic antidepressant. In terms of its mechanism of action, amitriptyline inhibits serotonin and noradrenaline re-uptake almost equally. A pain model that involved esophageal and rectal distension was not sensitive to amitriptyline[7]. This study applied a rather uncontrolled stimulation paradigm in which there was a risk for bias in stimulus intensity[9]. Hence, more studies are necessary to determine if the stimulation paradigm caused the lack of sensitivity.

Regarding the studies of ketamine and the antidepressants, more knowledge could be obtained on their effects by combining these experimental studies with studies on pharmacokinetics. However, experimental human pain studies often lead to new information, and as such studies often consist of a very complicated protocol and setup, it is not always possible to study the pharmacokinetic profile in parallel.

CONCLUSION

To improve visceral pain treatment, it is important to study the underlying physiological mechanisms of the pain and the pharmacological mechanism of action of the different analgesics. Experimental human visceral pain research bridges the knowledge gap between animal studies and clinical studies in patients suffering from pain, making it an important tool in translational pain research, as illustrated in Figure 1. An experimental pain model activates different modalities and, therefore, explores the effect of analgesics. With further understanding of the cerebral pharmacokinetics and pharmacodynamics of analgesics, opportunities may emerge to improve the efficacy and safety of these drugs in clinical practice. In combination with PK-PD studies and objective assessments such as EEG, new information regarding a given drug, its dose regimen and its effects can be obtained. Thus, evaluation of pharmacokinetics and pharmacodynamics is needed in future drug research. It is of interest to study the effect of new drugs as well as drugs already on the market, as lack of knowledge on the pharmacokinetics and pharmacodynamics of analgesic agents makes treatment of visceral pain a difficult task, and often far from optimal.

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GUIDELINES CLINICAL PRACTICE

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New technologies to investigate the brain-gut axis

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Abstract

Functional gastrointestinal disorders are commonly encountered in clinical practice, and pain is their commonest presenting symptom. In addition, patients with these disorders often demonstrate a heightened sensitivity to experimental visceral stimulation, termed visceral pain hypersensitivity that is likely to be important in their pathophysiology. Knowledge of how the brain processes sensory information from visceral structures is still in its infancy. However, our understanding has been propelled by technological imaging advances such as functional Magnetic Resonance Imaging, Positron Emission Tomography, Magnetoencephalography, and Electroencephalography (EEG). Numerous human studies have non-invasively demonstrated the complexity involved in functional pain processing, and highlighted a number of subcortical and cortical regions involved. This review will focus on the neurophysiological pathways (primary afferents, spinal and supraspinal transmission), brain-imaging techniques and the influence of endogenous and psychological processes in healthy controls and patients suffering from functional gastrointestinal disorders. Special attention will be paid to the newer EEG source analysis techniques. Understanding the phenotypic differences that determine an individual's response to injurious stimuli could be the key to understanding why some patients develop pain and hyperalgesia in response to inflammation/injury while others do not. For future studies, an integrated approach is required incorporating an individual's psychological, autonomic, neuroendocrine, neurophysiological, and genetic profile to define phenotypic traits that may be at greater risk of developing sensitised states in response to gut inflammation or injury.

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Key words: Brain-gut axis; Central processing; Neuraxis; Neurophysiology

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INTRODUCTION

Pain is a complex multidimensional experience comprising sensory-discriminative, affective-motivational and cognitive-evaluative components[1]. The sensory-discriminative component represents the ability to localise pain, and assess its intensity whereas the affective-motivational component qualifies its unpleasantness and gives rise to emotional aspects such as fear and distress. The cognitive-evaluative component allows the evaluation and interpretation of the pain experience and is involved in attention, anticipation and memory of the experience[2].

Pain is an extremely common symptom in clinical practice[3] and often emanates from the intra-abdominal viscera. Visceral pain can be the manifestation of a myriad of underlying pathologies, occur with varying intensities ranging from mild discomfort to severe pain, be acute or chronic, and be referred to a variety of locations such as the chest, pelvis and skin. Understanding the complex mechanisms leading to the development and maintenance of visceral pain, in particular that which arises from

the gastrointestinal tract, requires an appreciation of the neuroanatomical structures and neurophysiological processes involved. The gastrointestinal (GI) tract has a complex innervation including sensory neurones (afferents), and the rich neuronal innervation closely regulates visceral function as well as providing sensory information to higher structures. The ability to dissociate specific neurophysiological mechanisms of aberrant gastrointestinal sensory processing has been the aspiration of an increasing number of gastrointestinal researchers. Improved access to brain imaging techniques has vastly increased our understanding of the central processing of gastrointestinal sensation and pain in both healthy volunteers as well as in patients suffering from functional gastro-intestinal disorders (FGID).

So how far are we now? As the episodical gastrointestinal pain still exploits different non-investigated aspects, the question is whether the newer brain-imaging techniques have provided the scientists with further understanding of the underlying pathophysiology and mechanisms in FGID? This review will focus specifically on the sensory pathways (peripheral, spinal and supraspinal) involved in these pain mechanisms and highlight the newer techniques in electroencephalogram (EEG) source analysis.

SENSORY INNERVATION OF THE GASTROINTESTINAL TRACT

The gastrointestinal (GI) tract has a complex innervation with sensory neurones (afferents). As well as receiving dual sensory innervation from the central nervous system (CNS) referred to as extrinsic afferents, it has its own integrated network of intrinsic afferents (the enteric nervous system, ENS), that project locally. This rich neuronal innervation closely regulates visceral function as well as providing sensory information to higher structures.

Intrinsic sensory innervation (enteric afferent neurones)

The hollow intra-abdominal viscera have a rich sensory innervation with locally projecting afferent neurones, forming the enteric nervous system, whose cell bodies are located in the myenteric or submucosal plexuses[4]. This network of neurones and interneurones has a structural complexity and functional heterogeneity similar to that of the CNS, but mainly regulates local functions and reflexes such as secretion, motility, mucosal transport and blood flow[5,6]. Motor neurones located within the ganglia of the ENS coordinate these functions largely by regulation from local sensory neurones, although some also receive inputs from the CNS *via* autonomic (both sympathetic & parasympathetic) pathways[7]. Although the majority of enteric afferent axons are confined to the gut wall, some can project to the pre-vertebral ganglia of the sympathetic nervous system[8].

Extrinsic sensory innervation (primary afferent neurones)

The gastrointestinal tract has a dual sensory innervation from the CNS. In humans, visceral afferents project to the CNS mainly *via* the vagus nerve to the brainstem (vagal afferents) or through splanchnic nerves to the spinal cord (spinal afferents), and are described below.

Vagal afferent neurones

The vagus nerve innervates the majority of the GI tract apart from the distal third of the colon[9]. 70%-90% of the fibres in the vagal trunks are unmyelinated C-fibre neurones with their cell bodies located in the nodose ganglia situated just below the jugular foramen, although a minority lie more proximally within the jugular ganglia and contain afferents primarily from the oesophagus[10]. Around 80%-85% of nerve fibres in the vagus are afferent and project viscerotopically to the medial division of the nucleus of the solitary tract (NTS). Second-order neurones project from the NTS to sites in the brainstem, hypothalamus and amygdala including the vagal motor nuclei, the rostral areas of the ventrolateral medulla and the parabrachial nuclei[11,12]. Cortical projections from the brainstem include the orbitofrontal, infralimbic anterior cingulate and insula cortex, the latter having reciprocal connections with the secondary somatosensory cortex.

Vagal afferents are classically believed to mediate non-noxious physiological sensations such as satiety and nausea due to their low response thresholds and saturation characteristics that are within the physiological range[13-15]. However, animal experiments have suggested that vagal afferents may be involved in the central inhibitory modulation of pain. For instance, electrical stimulation of cervical vagal afferents inhibits the responsiveness of spinothalamic tract neurones to noxious stimuli[16].

Spinal afferent neurones

Spinal afferent neurones project from the viscera through the splanchnic nerves to the thoracic, upper lumbar and sacral spinal cord with their cell bodies located in the dorsal root ganglia (DRG). They constitute only 5%-10% of all afferent fibres in the thoracic and lumbar dorsal nerve roots with the majority traversing the pre- and paravertebral ganglia en route to the spinal cord. Collaterals to the prevertebral ganglia may mediate local autonomic reflexes[7].

Spinal afferents are contained within the cardiac (superior, middle and inferior) and splanchnic (thoracic, greater and lesser) nerves. These pass through the white rami to join spinal nerves before entering the DRG. The oesophagus is innervated craniocaudally by afferents from the DRG located between the first cervical and third lumbar segments. Retrograde labelling studies have shown the maximum distribution of spinal sensory neurons to be in the following DRG: C1-T8 (striated muscle); C5-L2 (smooth muscle), and T1-L3 (lower oesophageal sphincter)[17].

SPINAL PAIN PROCESSING

From the cell bodies within the DRG, spinal visceral afferents enter the spinal cord and ascend or descend one or two spinal levels in the dorsolateral fasciculus (Lissauer's tract) before terminating within the grey matter. In the

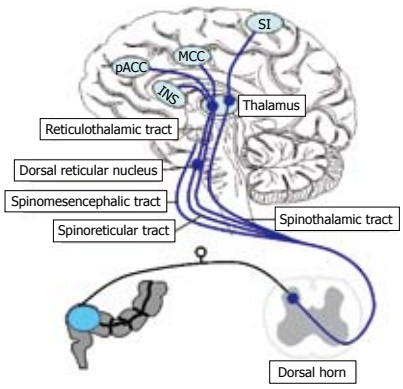

Figure 1 The principal visceral projections from the spinal cord to subcortial and cortical structures (blue lines). The spinothalamic tract terminates in the medial and posterior thalamus. Thalamocortical fibres then project to the primary somatosensory cortex. The spinoreticular tract terminates in the reticular formation to the medial thalamus. The spinomesencephalic tract projects to various regions in the brainstem, including the periaqueductal grey, locus coeruleus, and dorsal reticular nucleus in the medulla. Thalamocortical projections from the medial thalamus project to the cingulate cortex and insula which are involved in processing noxious visceral and somatic information. The brain regions innervated by these pathways that respond to painful visceral stimuli include the thalamus, insula, amygdala and anterior cingulate cortex (ACC). The ACC is comprised of two components, the perigenual ACC (pACC) involved in affect and the mid cingulate cortex (MCC) with behavioural response modification. Other pathways for transmission of noxious visceral stimuli (such as the dorsal column pathway), exist, but are not shown here.

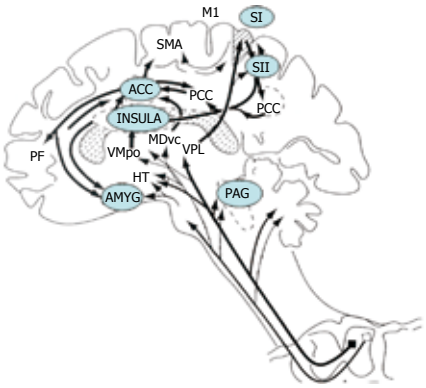

Figure 2 The subcortical and cortical structures that have been shown to be activated in response to visceral pain. PAG: Periaqueductal grey; PB: Parabrachial nucleus of the dorsolateral pons; VMpo: Ventromedial part of the posterior thalamic nuclear complex; MDvc: Ventrocaudal part of the medial thalamic dorsal nucleus; VPL: Ventroposterior lateral thalamic nucleus; ACC: Anterior cingulate cortex; PCC: Posterior cingulate cortex; HT: Hypothalamus; S1, S2: First and second somatosensory cortical areas, respectively; PPC: Posterior parietal complex; SMA: Supplementary motor area; AMYG: Amygdala; PF: Prefrontal cortex; M1: Motor cortex. (Adapted from Price DD. Psychological and neural mechanisms of the affective dimension of pain. *Science* 2000; 288: 1769-1772).

1950's Rexed divided the spinal grey matter into a system of ten laminae (LI-LX) which in turn divides the grey matter into four regions: the dorsal horn (LI-VI), the intermediate zone (LVII), the ventral horn (LVIII and IX) and the region of the central canal (LX)[18]. Second order neurones in the afferent pathway have a cell body in the dorsal horn of the spinal cord and relay signals to the brain *via* a number of ascending tracts.

The central pathways for processing nociceptive information begin at the level of the spinal cord dorsal horn. Spinal afferent projections terminate in distinct laminae of the spinal cord dorsal horn (mainly I and V, and occasionally to the contralateral laminae V and X) where they are organised in a segmental manner, but distributed over several spinal segments[19]. This diffuse termination pattern may explain the poor localisation of visceral sensation often seen in clinical practice, whereas the convergence of visceral and spinal afferents in the spinal dorsal horn may explain the phenomenon of viscerosomatic convergence, whereby visceral pain is often referred to nearby somatic structures[20,21].

ASCENDING SPINAL PATHWAYS

The ascending spinal tracts that convey sensory information to supraspinal structures are contained within the anterior lateral and posterior tract systems. The anterior lateral system comprises the spinothalamic, spinoreticular, spinomesencephalic, and spino-limbic tracts, illustrated in Figure 1. The medial and lateral subdivisions of the spinothalamic tract project to the medial/intralaminar and ventral/ventral posterior lateral (VPL) nuclei of the thalamus. respectively[22]. Third-order thalamocortical fibres then project to the somatosensory, insula and medial prefrontal cortices[23]. The spinothalamic tracts mediate sensations of pain, cold, warmth and touch and are also important for sensory discrimination and localisation of visceral and somatic stimuli[24,25].

The spinoreticular tract conducts sensory information from the spinal cord to the reticular formation in the brainstem. The reticular formation is mainly involved in the reflexive, affective and motivational properties of such stimulation[26]. Third-order reticulothalamic tract neurons project from the dorsal and caudal medullary reticular formation to the medial and intralaminar nuclei of the thalamus. From the intralaminar nuclei, ascending pain signals spread bilaterally to the prefrontal cortex (PFC), including the anterior cingulate cortex (ACC)[25]. The spinomesencephalic tract ascends the spinal cord with fibres to various regions in the brain stem, including the periaqueductal grey (PAG), locus coeruleus (LC), and dorsal reticular nucleus in the medulla[25].

The spinolimbic tracts project to areas such as the amygdala, medial thalamus, hypothalamus and other limbic structures, and are also believed to be important in mediating the motivational aspects of pain[25]. See Figure 2.

The posterior system comprises three synapsing tracts: first order dorsal column neurones, the post-synaptic dorsal column (PSDC) pathway and the spinocervical tract. These pathways were not believed to convey nociceptive information; however, recent studies have highlighted the importance of the dorsal column in viscerosensory processing. Al-Chaer demonstrated in primates that the responsiveness of neurones in the ventral posterior lateral nucleus of the thalamus to colorectal distension could be significantly attenuated by dorsal column lesions[27]. Lesions of other tracts had no consistent effects, thus, supporting the role of the dorsal column in conveying visceral nociceptive input to the thalamus.

PAIN PROCESSING IN THE BRAIN

Knowledge of how the brain processes sensory information from visceral structures is still in its infancy; however, our understanding has been propelled by technological imaging advances such as functional Magnetic Resonance Imaging (f-MRI), Magnetoencephalography (MEG), Positron Emission Tomography (PET), and EEG. Human studies have non-invasively demonstrated the complexity involved in pain processing, and highlighted a number of subcortical and cortical regions involved.

The pathways involved in the perception of visceral pain are highly complex. In addition, these pathways are dynamic and amenable to change in response to internal or external stressors. Numerous mechanisms can be engaged in response to stressors from the primary afferent level right up to the cerebral cortices, resulting in a high degree of plasticity in the nervous system. The ultimate outcome of pain perception is brought about by a delicate balance between facilitatory and inhibitory mechanisms. As pain is a conscious feeling, the ultimate goal in pain-imaging is to follow the pain stimulus throughout the neuraxis.

Imaging studies have been performed to explore normal brain processes involved in visceral perception, whether liminal or subliminal and its modulation by attention, conditioning and emotion[22,28-31]. Several studies have also looked at the role of visceral perception in emotions and cognitive processes such as learning[32,33].

Visceral pain has been contrasted with pain arising from superficial skin structures[34,35]. Recent reviews have summarized imaging findings in normal GI sensation[36-38].

Recently, a number of new technologies have emerged within imaging of the brain-gut axis, and in this review we focus on the EEG techniques where signal analyses have made it possible to follow the early and pain specific pathways to the brain with high temporal and spatial resolution.

IMAGING TECHNIQUES

Most commonly f-MRI is based on a technique using different paramagnetic properties of oxy- and deoxyhaemoglobin in the blood. These regional changes in blood flow, volume and oxygenation of haemoglobin derive from changes in neuronal activity and, thus, regions of activation may be identified by subtracting regional cerebral blood flow during a control condition from blood flow during a stimulus condition or by correlating regional blood flow with the intensity or time course of a stimulus or its perception[2]. A major advantage of f-MRI is that it is non-invasive and non-cumulative, allowing subjects to be studied repetitively. f-MRI has an excellent spatial resolution (2-5 mm), especially in the more superficial layers. Limitations are seen in the deeper structures, such as the brainstem and thalamus, due to pulsation artefacts. The temporal resolution is poor (1-3 s) and therefore f-MRI is not a specific tool for investigating the neuronal activity directly related to the painful stimuli. Since the exogenous brain activity takes place within the first 150 ms post stimulus, the response may miss the fast occurring activity and model, instead, the endogenous activity rather than brain responses due to pain. In contrast to PET studies a limitation in f-MRI studies is the lack of information regarding neurotransmitters or involved receptors[39]. A comparison between localization of visceral and somatic regions of the oesophagus in healthy subjects using fMRI has been done[40]. Distension of the distal oesophagus was represented bilaterally at the junction of SI and SII. Different activation patterns were also observed in the ACC, prefrontal cortex and cerebellum. Another recent study was carried out to determine whether behavioural differences are due to differences in the central processing of visceral and somatic pain[30]. It was demonstrated that visceral stimuli induced deactivation of the perigenual cingulate bilaterally with a relatively greater activation of the right anterior insula i.e. regions encoding affect. Kwan *et al*[41] used f-MRI as a diagnostic tool for demonstrating abnormal brain processing in Irritable Bowel Syndrome (IBS). They identified abnormal event-related sensations in five brain regions following rectal distensions. In the primary sensory cortex, there were urge-related responses in the IBS, but not the control group. In the medial thalamus and hippocampus, there were pain-related responses in the IBS, but not the control group. However, pronounced urge- and pain-related activations were present in the right anterior insula and the right anterior cingulate cortex in the control group, but not the IBS group. These findings conflict with the findings of Bonaz *et al*[42], who demonstrated significant deactivations within the right insula, the right amygdala, and the right striatum following rectal stimulations in patients suffering from IBS compared to healthy subjects.

PET

PET measures the cerebral blood flow after injection of a radioisotope. The most commonly used in gastrointestinal research is $H_2{}^{15}O$ labelled water. PET has excellent spatial resolution (2-5 mm) and allows the operator to tag important biological molecules that bind to targeted receptor groups or glucose metabolism in active neuronal tissue. PET is superior in imaging radiopharmaceuticals and/or other ligands as it offers the ability to study receptor distribution and explore the site of action[2]. However, the temporal resolution is poor (minutes), and as the subject receives a considerable dose of radiation, group analyses are needed for meaningful results, interpreting endogenous brain activity following pain rather than exogenous brain activity following painful stimulation. Another major disadvantage is the expense of a PET scanner.

Silverman *et al*[43] characterized the cerebral processing of visceral noxious events, by measuring the changes in regional cerebral blood flow. Healthy controls demonstrated a significant increase in anterior cingulate cortex activity following noxious stimuli, whereas no activity was seen in response to non-painful stimuli. In patients suffering from IBS, the ACC failed to respond to the same stimuli, whereas significant activation of the left prefrontal cortex was seen. In contrast, another study

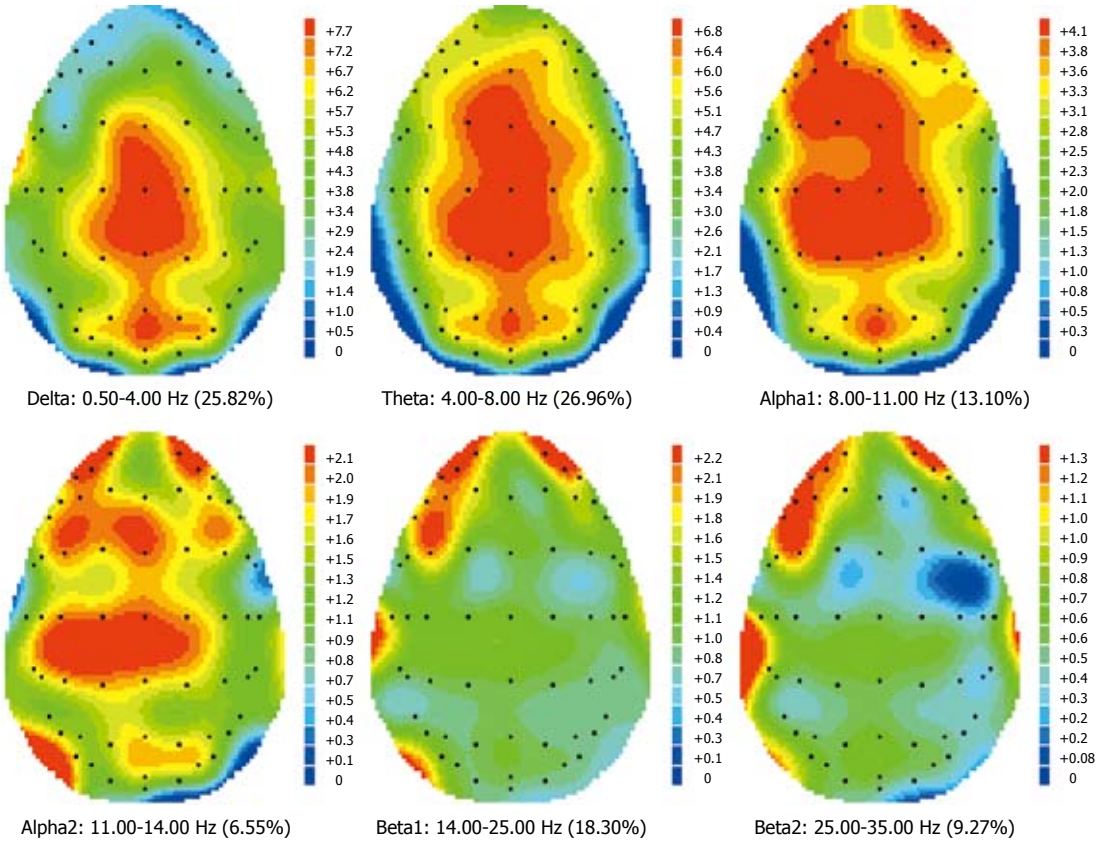

Figure 3 Example from painful CEP from the gut performed in a healthy volunteer. The figure shows the topographies at different frequency bands from one subject, and the percentage of the presence of each frequency band in the overall signal. The black dots represent electrodes. The colours represent how much power a particular frequency band holds at each electrode. The scales describing the colours are to the right of the topographies.

compared healthy controls and patients suffering from IBS, and found no group differences in anterior insula and dorsal anterior cingulate cortex (dACC) activity, two regions consistently activated by painful intestinal stimuli[44]. However, IBS patients showed greater activation of the amygdala, rostroventral ACC, and dorsomedial frontal cortical regions.

MEG

MEG is a non-invasive brain imaging tool, which allows detection of cortical neuromagnetic activity as opposed to metabolic changes, which are secondary. The spatial resolution is comparable to f-MRI and PET; however, MEG also has millisecond temporal resolution, and is suitable for both individual and group studies. MEG is not widely available; systems are only present in specialist centres. The technical limitation of MEG is that it is less able to resolve the radial current, and is not sensitive to deep sources; but it is especially sensitive to the tangential activity in the cortex.

EEG

EEG measures direct electrical brain activity, through non-invasive scalp electrodes. This electrophysiological tool is widely used. EEG can be used to investigate the activity in both health and disease, as it is non-invasive and completely harmless. While f-MRI and PET brain imaging techniques have excellent spatial resolution, their time resolution is poor. Thus, these methods do not directly show brain activity in time. The EEG signal is divided into five frequency bands: Delta: < 4 Hz, Theta: 4-8 Hz, Alpha 8-12 Hz, Beta: 13-30 Hz, and Gamma: greater than 30 Hz. Figure 3 shows an example of a presentation of different frequency bands present in a painful cortical evoked potential (CEP) in the oesophagus. Analyses like this can be used to compare frequency alterations and topographical appearance between different subject groups. Drewes *et al*[45] found significant differences in theta and delta bands in CEPs between healthy controls and patients with chronic pancreatitis (CP) following painful stimulation in the gut. The patients showed higher activity in the theta band and the main theta band components oscillated by 4.4 Hz in patients and by 5.5 Hz in controls. Furthermore, the energy in the delta band was higher in the controls, whereas patients only showed scattered delta activity.

EEG recordings can be used for CEP, which detect brain activity in real time, with temporal resolution on the millisecond scale. CEP is an electrical response in the brainstem or cerebral cortex following a stimulus, i.e. painful stimulation in the gut. CEP amplitudes are typically lower than the amplitudes of spontaneous EEG (less than a microvolt to several microvolts, compared to tens of microvolts for EEG); but, since the CEPs are time-locked to the stimulus and the background activity occurs randomly, the CEP amplitudes become higher during the averaging process, and most of the background noise cancels out. In order to extract the CEPs with a

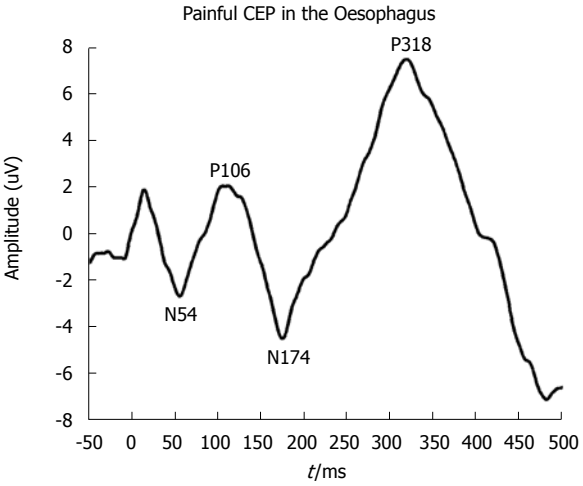

Figure 4 CEP at Cz electrode from a healthy volunteer. The subject was electrically stimulated in the oesophagus through a 6-mm nasal endoscope. Electrical stimulation was at the pain threshold and this CEP is an average of 35 such stimulations.

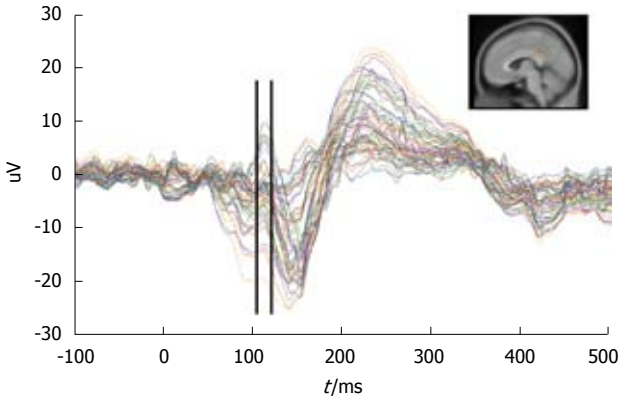

Figure 5 This is a butterfly plot of 62 channels (data of all 62 channels superimposed on each other). The vertical lines mark the time course of the peak that was used for analysis. The red dot on the MRI image on the top right corner represents where the activity was calculated to be by MUSIC.

good signal-to-noise ratio, a number of stimulations are presented at a certain frequency, and these stimulation trials are then cleaned for artefacts and averaged. An example of an averaged painful CEP from electrical stimulation of the gut is shown in Figure 4. Each peak in the CEP represents a synaptic event associated with the synchronous transmission of afferent information from one group of neurons to another. Several studies have examined the amplitudes and latencies of painful CEPs in the gut, and compared the results between a control group, and a study group (i.e. patients suffering from chronic pancreatitis, non-cardiac chest pain or patients treated with analgesics)[46-51]. Dimcevski *et al*[46] showed decreased early CEP latencies in patients with CP compared to healthy controls. Sami *et al*[48] showed decreased latencies in the first two positive peaks (P1 and P2) of CEPs following painful stimulation in the oesophagus after acid perfusion. Rossel *at al*[47] found that P1 had a shorter latency and smaller amplitude in patients with IBS compared to healthy controls. Furthermore, the group showed that the controls had a mid-latency positive component after 100 ms, which was absent in the patient group, and the healthy controls had a single late positive component (> 150 ms) whereas the IBS group had a late component which was biphasic. The demonstrated changes in latencies and frequencies most likely explain neuronal changes, such as plasticity, in the CNS.

INVERSE MODELLING OF CORTICAL EVOKED POTENTIALS

EEG is a mixture of signals from all over the brain due to the current generated by groups of neurons not only being produced at the source location, but also flowing to the surrounding tissue *via* volume conduction. Thus, by the time the signal arrives at the scalp electrodes it is distorted. Therefore, while CEPs have excellent time resolution on the millisecond scale, the spatial resolution is limited, and it is impossible to predict which sources in the brain are generating these potentials. However, methods using advanced mathematics and signal analysis to address these problems exist. This is known as "inverse modelling." Inverse modelling is based on the idea that groups of neurons generating the potentials at the scalp can be modelled by equivalent current dipoles. From multiple-channel recordings of CEPs, it is possible to mathematically calculate the locations of these dipoles. In order to do this, freeware and commercial software such as [EEGLAB, BrainStorm, Statistical Parametric Mapping (SPM), BESA, ASA and CURRY] are available. Some studies have performed inverse modelling on CEPs following painful stimulation in the gut. Dimcevski *at al*[46] found that dipolar activities corresponding to the early CEPs were located consistently in the bilateral insula, in the anterior cingulate gyrus, and in the bilateral secondary somatosensory area. Furthermore, they showed that in a CP patient group, the bilateral insular dipoles were localized more medial than in the healthy control group. They also showed changes in the cingulate cortex where the neuronal source was more posterior in patients than in controls. Drewes *et al*[52] showed two dipoles in the bilateral insular cortex, one dipole in the anterior cingulate gyrus and two dipoles in the bilateral secondary somatosensory area post the painful stimulus. Moreover, they found the anterior cingulate dipole to have a more posterior position in IBS patients than in healthy controls[53]. Inverse modelling algorithms, such as low-resolution brain electromagnetic tomography (LORETA) and multiple signal classification (MUSIC) have usually been applied to instantaneous CEP data by selecting a certain time frame in the data and calculating the location of dipole(s) generating the CEP at this time, see Figure 5.

Different inverse modelling algorithms and the ideas behind them are discussed in detail elsewhere[54-57]. The disadvantage of performing inverse modelling on instantaneous CEPs is the instability of algorithms when multiple sources are active and the interference of background electrical and physiological noise. For this reason, different signal decomposition methods have been used in order to separate the signal into a sum of

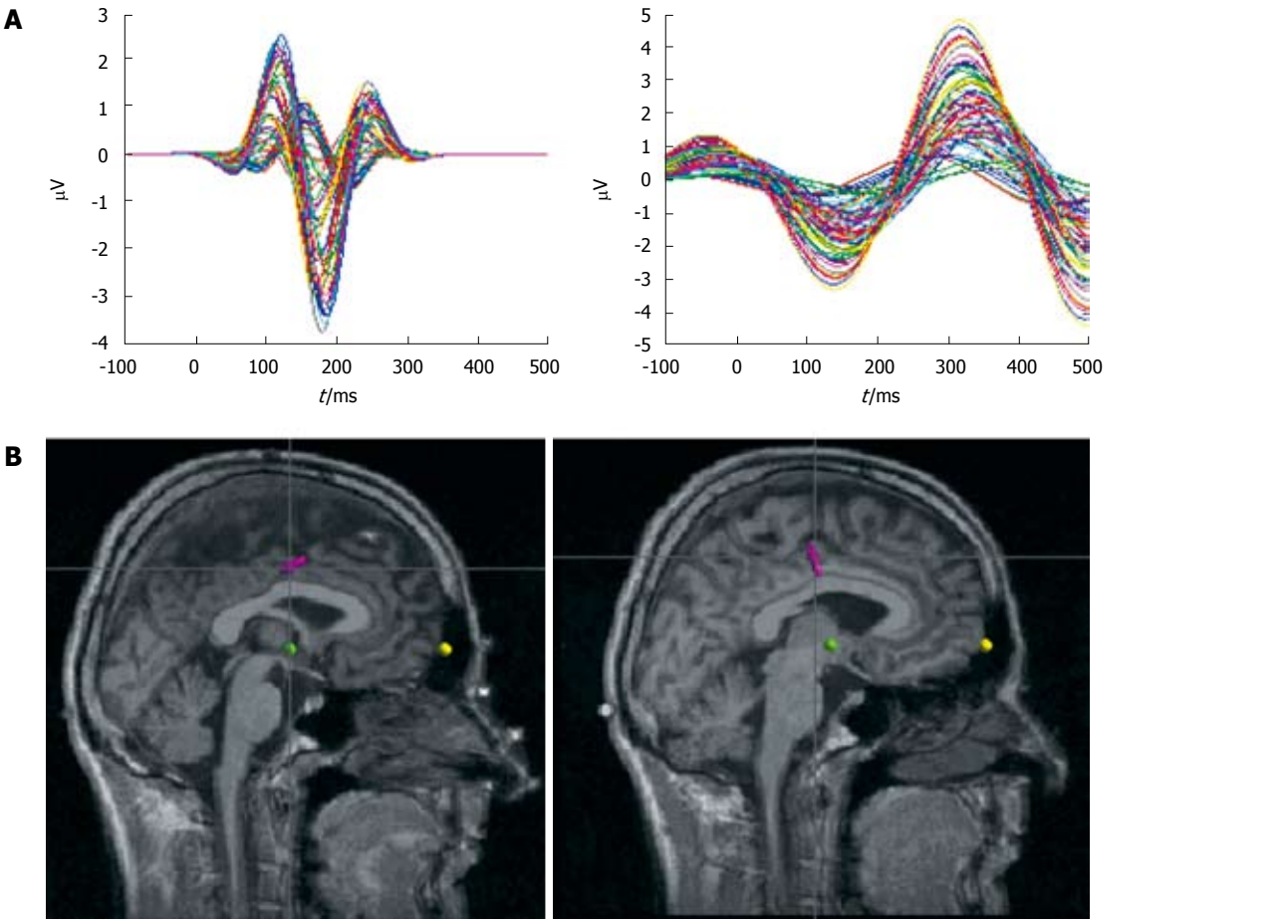

Figure 6 An example of two MMP atoms from painful CEPs in the oesophagus. A: Butterfly plots of the atoms; B: Dipole location of each atom.

waveforms, each having a single dipole generator. These methods make it possible to differentiate signals corresponding to brain activity from those corresponding to noise and artifacts. Once the signals are decomposed, inverse modelling can be completed on each waveform, and furthermore, it is possible to observe at which time and frequency this particular dipole is active, as shown in Figure 6.

Recently, Multichannel Matching Pursuit (MMP) was introduced, which decomposes the data into a sum of waveforms (usually termed atoms), each of them defined in time, frequency and space. We showed that decomposing the CEPs using MMP prior to inverse modelling (namely MUSIC) is superior to some blind source separation (BSS) methods, namely Independent Component Analysis (ICA) and Second-Order Blind Identification (SOBI), which are typically used for CEP signal decomposition prior to source analysis. These decomposition methods are described in detail elsewhere[58-66]. Additionally, we showed that MMP prior to MUSIC was much more accurate than MUSIC on the instantaneous data on both simulated and empirical CEPs[67]. MUSIC on MMP atoms was able to localize deep, superficial, and simultaneously active dipoles with high accuracy. The spatial resolution for MUSIC on MMP atoms was 3-20 mm compared to MUSIC on ICA components (5-27 mm for superficial dipoles, deep dipoles failed to localize), MUSIC on SOBI components (5-32 mm, deep dipoles failed to localize), and MUSIC on raw data (7-81 mm, simultaneously active dipoles typically did not localize correctly). Comparisons between different inverse modelling methods have been carried out in other studies[54,55]. We chose MUSIC because it has demonstrated an advanced ability to localize a restricted number of independent sources, and has the ability to reliably replicate temporal waveforms[57]. Furthermore, it is possible to combine an individual's MRI scan with the digitized locations of electrodes on their scalp in order to create a realistic head model, and use this head model to find the inverse solution for the individual's CEPs. These combinations of non-invasive methods allow us to study the sequence of cortical activations due to pain. Although combination of MMP, inverse modelling, and individual MRIs allows us to find new information regarding pain processing in the brain, one shortcoming of MMP is the lack of order in the atoms. This makes it difficult to compare between groups; hence, to distinguish which atoms from one subject correspond to the atoms of another subject and which atoms in one group are different/similar to the atoms in another group. For this reason, clustering of atoms/dipoles can be done. Delorme *et al*[58] have implemented such a method for clustering of ICA components and incorporated it into their EEGLAB toolbox. Currently, we are developing a toolbox to cluster MMP atoms based on time/frequency, topography, both time/frequency and topography, or dipoles. Furthermore, it is

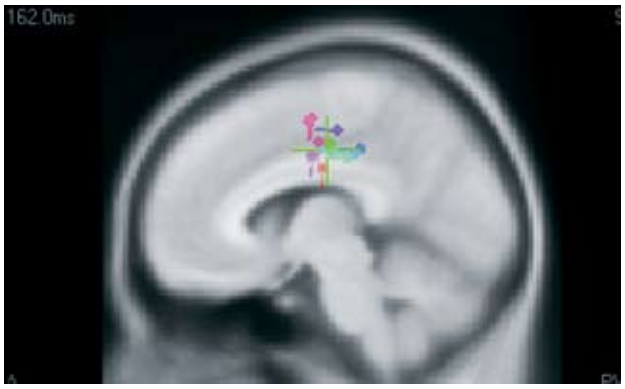

Figure 7 One of the clusters of cingulate dipoles generating CEPs following a painful stimulation in the gut in 10 subjects.

possible to take the Talairach coordinates of each dipole in individual clusters, and look up the anatomical location of the source in a Talairach atlas. For an example of clusters, see Figure 7. These dipoles were localized in the cingulate gyrus. In the future, we are aiming at clustering the dipoles after source localization has been performed using realistic head models for each individual (i.e. combining their individual MRI scans and digitized EEG electrode locations). These methods will allow for more precise source localization, automated separation of dominant sources during painful CEPs in different groups, and allow the study of the sequential order of activated centres post stimulus. These advancements will provide new insight into how different subject groups process pain.

THE BRAIN-GUT AXIS IN FUNCTIONAL GASTROINTESTINAL DISEASES

The ROME II multinational consensus has defined functional gastrointestinal disorders (FGID) as "a variable combination of chronic or recurrent gastrointestinal symptoms which are not explained by structural or biochemical abnormalities"[68]. Despite remarkable advances in our understanding and management of "organic" gastroenterological complaints such as peptic ulcer disease, IBS and even cancer over the last 30 years, our understanding of the mechanisms of pain in FGID patients remains far from complete. The lack of effective treatments for these disorders leads to chronic symptoms, recurrent attendances in hospital, poor patient satisfaction and significant morbidity. Health care costs are estimated to be around $34 billion in the 7 largest Western economies[69,70].

Although patients with FGID show marked heterogeneity in their clinical presentation and response to treatment, common features have become apparent as our knowledge of these disorders has increased. These patients often display a heightened sensitivity to experimental gut stimulation, termed visceral pain hypersensitivity (VPH) which is believed to be important in their pathophysiology and symptom generation. The hypersensitivity may be caused by peripheral and central factors relating to primary afferents as well as the autonomic and enteric nervous systems; however, in this review we will focus on the changes in the CNS which can be elucidated using the new imaging techniques described above.

Mayer *et al*[71] studied the perceptual responses to rectosigmoid distension in IBS patients and controls with functional brain imaging using $H_2{}^{15}O$ PET and found that following a train of repetitive sigmoid distensions, control subjects demonstrated greater activation of the PAG and thalamic regions compared to patients. This effect was seen both during actual rectal distension and during expectation of the stimulus, despite its absence. As has been outlined, the PAG is an important structure involved in the modulation of spinal pain processing, and the above finding suggests that a proportion of IBS patients have inadequate activation of brain regions involved with antinociception. Mayer *et al*[38] have recently reviewed imaging studies in FGID which has been critiqued by Hobson and Aziz[36,37,72].

"Visceral hypersensitivity" is a hallmark feature in IBS patients, who show an abnormal pattern of ACC activation during pain perception which is an interesting parallel to ACC activation relative to increasing pain perception in healthy subjects[43,73,74]; hemispheric preference, as well as cognitive style of information processing served as indicators of covert changes in brain functions in 21 adult IBS patients[75]; and abnormal cerebral processing of oesophageal stimuli was found in patients with noncardiac chest pain[50,51]. Drossman *et al*[76] found that alterations in brain activity were associated with resolution of emotional distress and pain in a case of severe IBS.

A recent longitudinal study in IBS found that there were significant decreases in amygdala, dACC and dorsal brainstem activation over a 12-mo period during anticipation for pain although pain-related activations and symptoms were stable[77]. Rectal pain induced significant activation of the perigenual ACC, right insula and right prefrontal cortex. Amitriptyline was associated with reduced pain-related cerebral activations in the perigenual ACC and the left posterior parietal cortex, but only during stress[78]. Taken together these findings strongly suggest that abnormalities in the brain-gut axis play a key role in our understanding of FGID, and future studies using the techniques described above will undoubtedly increase our understanding of these disorders.

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GUIDELINES CLINICAL PRACTICE

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New techniques in the tissue diagnosis of gastrointestinal neuromuscular diseases

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Abstract

Gastrointestinal neuromuscular diseases are a clinically heterogeneous group of disorders of children and adults in which symptoms are presumed or proven to arise as a result of neuromuscular (including interstitial cell of Cajal) dysfunction. Common to most of these diseases are symptoms of impaired motor activity which manifest as slowed or obstructed transit with or without evidence of transient or persistent radiological visceral dilatation. A variety of histopathological techniques and allied investigations are being increasingly applied to tissue biopsies from such patients. This review outlines some of the more recent advances in this field, particularly in the most contentious area of small bowel disease manifesting as intestinal pseudo-obstruction.

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Key words: Enteric myopathy; Enteric neuropathy; Interstitial cells of Cajal; Intestinal pseudo-obstruction; Visceral pain

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INTRODUCTION

The term gastrointestinal neuromuscular diseases (GINMD) describes a clinically heterogeneous group of disorders of children and adults in which symptoms are presumed or proven to arise as a result of neuromuscular (including interstitial cell of Cajal) dysfunction[1,2]. Common to most of these diseases are symptoms of impaired motor activity which manifest as slowed or obstructed transit[3] with or without evidence of transient or persistent radiological visceral dilatation. Such diagnoses include primary and secondary disorders of the oesophagus to the colon e.g. achalasia, gastroparesis, intestinal pseudo-obstruction and severe constipation. Pathologic abnormalities of the sensorimotor apparatus have been demonstrated in such disorders by a variety of methods since the 1960s; however, this remains an area of evolving interest especially with the increasing availability of newer techniques and more critical appraisal of those more established techniques.

This review outlines some of the more recent advances in this field, particularly in the area of small bowel disease manifesting as intestinal pseudo-obstruction. The area of Hirschsprung disease diagnosis, although numerically important (this being by far the most common GINMD) is not covered here since, although some contention exists, in general the techniques for this diagnosis are long and better established. The review covers the safe acquisition of tissue and advances in histopathological and allied techniques.

SAFE TISSUE ACQUISITION

Tissue may be taken with deliberate diagnostic intent or alternatively come as the by-product of emergency or planned surgical interventions. On this basis, tissues may take the form of mucosal, deep submucosal, seromuscular or full-thickness biopsies or resection

Figure 1 Laparoscopically-assisted full thickness jejunal biopsy. The port sites are shown. After finding a suitable proximal jejunal loop, the bowel is exteriorised by extending slightly the umbilical port incision and biopsy and suture closure performed extracorporeally (Courtesy of B Nyborg, Huddinge, Stockholm).

specimens. Of particular note are recent advances in minimally invasive surgery that have permitted safe access and biopsy of a variety of intra-abdominal tissues including full-thickness bowel biopsy[4]. In the context of GINMD, with some variations, the technique has now been applied to children with colonic dysmotility[5,6] and adults with small bowel dysmotility, predominantly those with proven chronic idiopathic intestinal pseudo-obstruction (CIPO)[7-10]. A very recent study reported on the safety and diagnostic yield of a predominantly laparoscopically-assisted approach (Figure 1) to biopsy the small and large bowel in a cohort of 124 adults with suspected GINMD from 3 European centres. Median operating time was 50 min, conversion rate was 2% and length of stay was 1 d. There was an 8% readmission rate for obstructive symptoms; however, other morbidity was minimal and there were no mortalities. Overall the specific diagnostic yield was 81%, being high for jejunal biopsies (89%), but low for a small number of ileal and colonic biopsies[10]. On this basis, an extracorporeal laparoscopically-assisted procedure appears safe and with acceptable yield if performed in the proximal small bowel for the indications in this study. Completely intracorporeal staple techniques may also be safe, but very little published data exists, at least for the jejunum[10]. Laparoscopic gastric biopsies may also now be taken at the time of gastric pacing[11], and may be important in predicting outcome from this procedure on the basis of ICC pathology (personal communication: Gianrico Farrugia).

The potential to increase yield with multiple biopsies must be balanced against the risk of complications. Clearly, whilst there is some evidence from colectomy and post-mortem small bowel that sections should be taken at fixed intervals to avoid missing 'patchy' abnormalities of muscle or nerve[12], extending this finding to suggest multiple biopsies, even with a small risk for each is not currently advised. On this basis, as well as the potentially increased risks of leakage, laparoscopic full-thickness colonic biopsy is currently not advised, although seromuscular biopsies have been shown to be safe in a large series of paediatric patients[6] and can also be used for determining the HSCR transition zone. The role of appendectomy as a diagnostic surrogate of GINMD has recently been suggested based on preliminary findings in diabetes[13], but needs further exploration[14]. The evolving technique of NOTES (natural orifice transluminal endoscopic surgery) will in the future (in the author's view) have an important role here, with proof of concept already demonstrated in the stomach[15]. Regardless of technique, because of regional differences, whenever full-thickness biopsies are taken, the corresponding intestinal segment(s) should be precisely indicated to the pathologist.

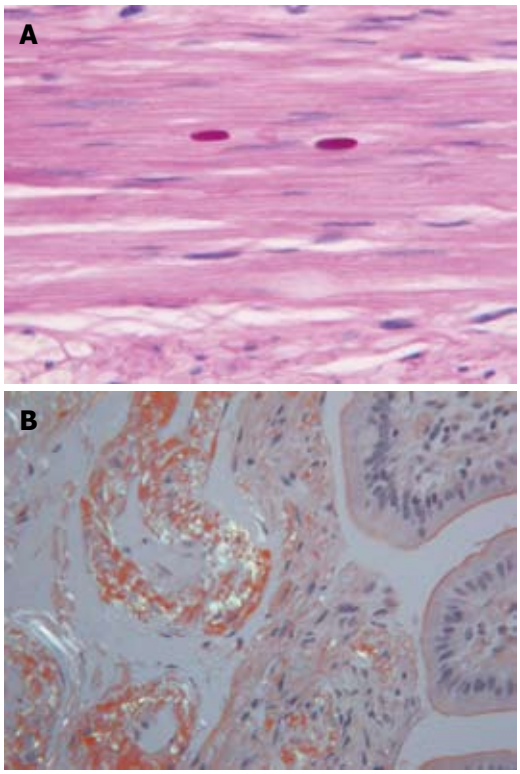

Figure 2 Tinctorial stains used in GI neuromuscular histopathology. A: Periodic acid Schiff staining showing polyglucosan bodies in a patient with intestinal pseudo-obstruction; B: Bifringence from amyloid visualised by Congo red staining (x 25-40).

HISTOLOGICAL TECHNIQUES

Although the histopathological diagnosis of GINMD (and exclusion of other disease) continues to be primarily based upon the analysis of H&E-stained sections with light microscopy, a number of other techniques can also be employed. A critical appraisal of the role of these techniques, particularly in comparison with the 'yield' of H&E, and guidelines for their use is currently being produced by an international working party: www.gastro2009.org/pdf/wp_project_descr07.pdf and is not covered here. Rather, descriptions of some newer diagnostic techniques are presented.

Tinctorial stains (Figure 2)

Although there is vast variation in current practice, tinctorial stains can supplement H&E with particular use in the assessment of specific structures and

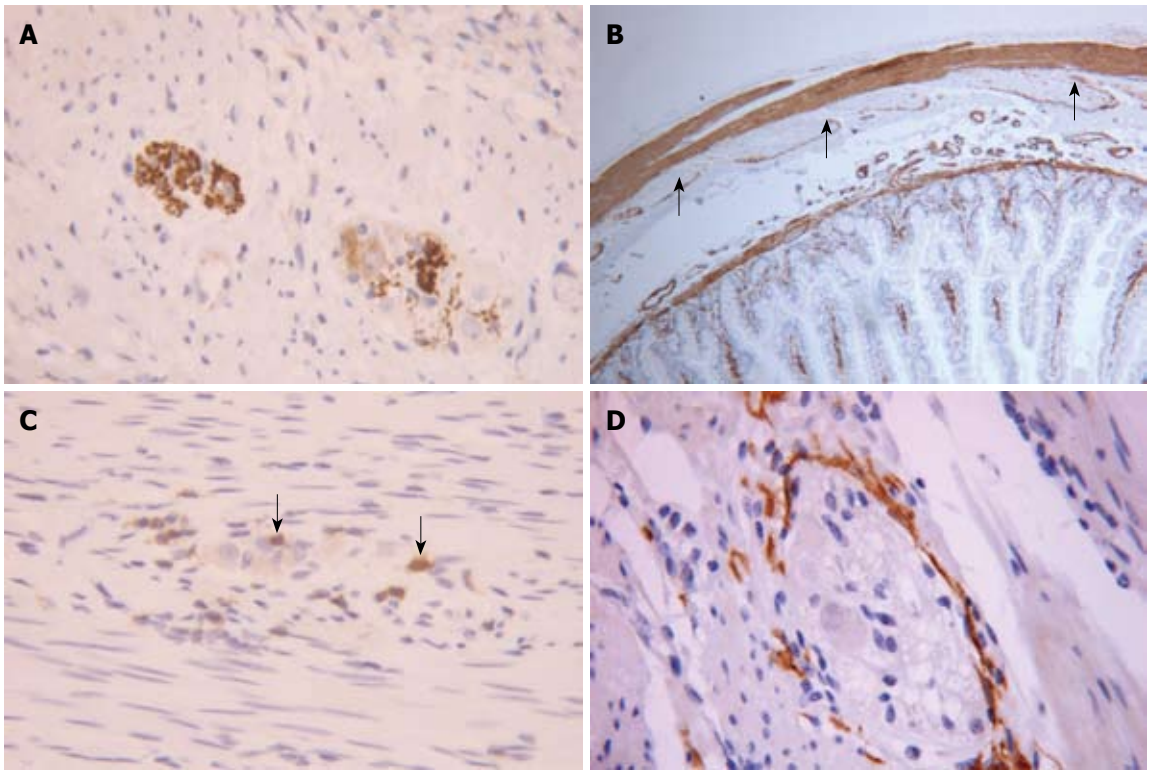

Figure 3 Immunohistochemistry using antibodies to. A: Neuron specific enolase allowing clear visualisation of myenteric ganglia, neuronal number and size; B: Smooth muscle alpha actin showing absent staining in the circular muscle layer of the jejunum (arrows) in a patient with enteric dysmotility; C: CD3 showing small numbers of periganglionic T lymphocytes (arrows) in numbers that most would deem abnormal and indicative of ganglionitis; D: CD117 staining showing normal myenteric plexus interstitial cells of Cajal (ICC-MP). (Original magnification x 40-100).

cell types. With periodic acid Schiff (PAS) staining, inclusion bodies e.g. polyglucosan, lipofuscin granules (secondary autophagic lysosomes), and glycogen can be observed, and PAS combined with diastase treatment can differentiate between glycogen and other structures (glycogen disappears after diastase pretreatment), which may be of value where a glycogenosis or related metabolic disorder are suspected. Polyglucosan inclusion body myopathy has recently been described in GINMD[16] and cannot easily be identified without use of PAS staining. Amyloid is a rare secondary cause of GINMD and can be detected with ease using Congo red staining. With Giemsa staining, mast cells and eosinophils can be seen easily, and the condition of the neuronal cytoplasm assessed (marginalization of the Nissl and chromatolysis). Various types of trichrome staining assist in the establishment of fibrosis and in differentiation from interstitial oedema (both cause increases in the distance between cells, and in early fibrosis this can be difficult to differentiate). Relevant to some rare cases of GINMD, Gomori trichrome staining is also used to diagnose mitochondrial neurogastro-intestinal encephalomyopathy (MNGIE) on the basis of finding 'ragged red fibres' on skeletal muscle biopsy[17].

Immunohistochemistry (IHC) (Figure 3)

The past thirty years has seen the use of IHC evolve in many areas of GI practice including that of GINMD diagnosis. With respect to mainly diagnostic rather than research applications, neuronal markers such as PGP9.5 and neuron specific enolase (NSE) may be employed to assist in the determination of neurons particularly if quantitation is considered important. This latter point is very contentious because heterogeneity of methods has meant that few normative data exist for any single method, especially when age and regional specificity are considered[18]. Nevertheless, diagnoses reliant on decreases in numbers of neurons and ganglia[19] have complemented findings made previously using the more laborious technique of silver staining[20]. Alpha smooth muscle actin deficiency has been demonstrated by IHC in some children[21] and adults[9] with GINMD, and stresses the importance also of regional specificity-this being a normal variant in the ileum[9]. Inflammatory neuropathies[8,10,22] and much less commonly leiomyopathies[23] may be best diagnosed by immunocyte IHC when large infiltrates (visible on H&E) are not apparent. This finding may prompt further autoimmune investigation (below) and dictate important changes in therapy[22-24]. Finally, c-kit (CD117) IHC has now become established in detecting changes in ICC numbers that certainly accompany, and may be causative of some GINMD[25].

Research applications of IHC have predominantly addressed disease mechanisms and pathways. In GINMD, many studies have attested to alterations in neurochemically-stained subsets of neurons allied to their differing functions. Changes said to underlie abnormal neuronal development[26], retarded colonic transit e.g. reduced substance-P[5], failure of sphincter

Figure 4 Electron micrograph of smooth muscle cells showing increased Golgi indicative of transition to a more secretory phenotype in a patient with enteric myopathy and pseudo-obstruction. (x 50 000).

relaxation e.g. decreased nitric oxide[27], or visceral hyperalgesia e.g. increased transient receptor potential channels[28] have been variously reported. Beyond mechanism and target identification, whether such changes may become clinical biomarkers of disease or guide treatment is the subject of much ongoing study, particularly if identifiable on endoscopic mucosal biopsy[29].

Electron microscopy (Figure 4)

Ultrastructural examination of neurons, muscle and interstitial cells of Cajal can be a useful adjunct to the above assessments in certain patients. These include some rare childhood myopathies where H&E findings are absent or equivocal (e.g. subtle fibrosis, atrophy of myocytes or myocyte vacuolation)[30], the identification of rare inclusion bodies[31] suggestive of mitochondrial disorders and some ultrastructural changes of ICC[32] and myocytes, including a transformation to more secretory phenotypes.

ADJUNCTIVE INVESTIGATIONS

Proteomic investigations

In cases characterized by clinical (adult onset, personal or family history of autoimmunity) and histopathological findings (especially inflammatory neuro or myopathies) an autoimmune pathogenesis may be suggested. A variety of antibodies directed to nuclear proteins[33,34] and, to a lesser extent, membrane-bound receptors[35,36] of the enteric neuromuscular compartment have been found in patients with secondary GINMP, especially of paraneoplastic origin. The presence of some of these autoantibodies in patients with idiopathic disorders affecting gut motility[22] has prompted their attempted identification in several recent studies[23,35,37,38]. In nearly all cases, proof of pathogenicity remains weak in comparison with established autoimmune diseases of the neuromuscular junction[39]. For a very recent full review, see Kashyap & Farrugia, 2008[40]. If clinically suspected, it is, however, reasonable to take a sample of serum for antibody testing. This should be sent to an established neuroimmunology unit where a variety of methods such as radioimmunoprecipitation assays may be employed[39] (Figure 5). Established antibody tests include those for anti-Hu[34] and anti voltage-gated calcium channels (particularly in paraneoplasia)[41], anti smooth muscle (particularly in myopathies and scleroderma), anti-ganglionic acetylcholine receptor (particularly if associated with dysautonomia)[35,39] and anti voltage-gated potassium channel antibodies[38,39]. One other blood-based investigation occasionally indicated in the investigation of pseudo-obstruction is the thymidine phosphorylase leucocyte activity assay[42] in patients suspected on the basis of clinical findings to have MNGIE[17].

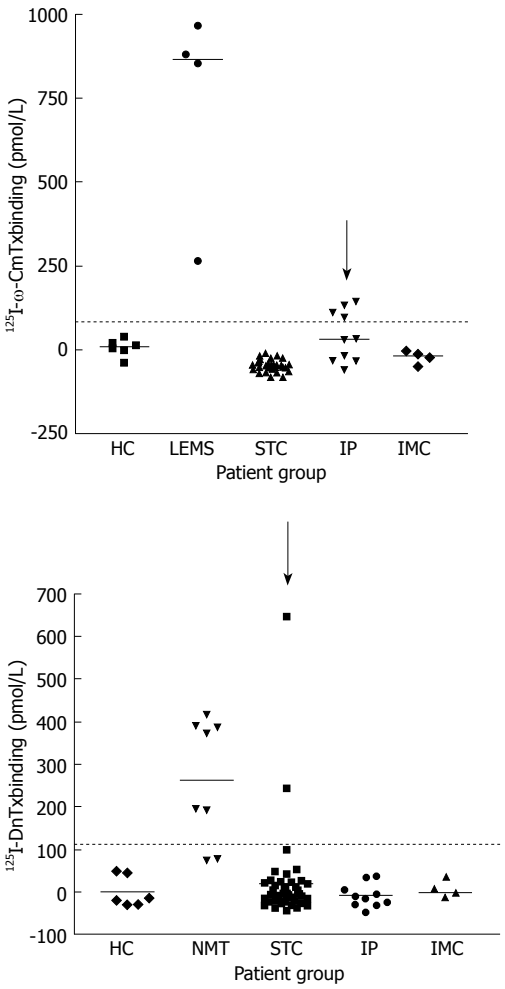

Figure 5 Radioimmunoprecipitation assays of sera from patients with GINMD and negative and positive controls. Assays for antineuronal antibodies directed to anti-voltage-gated calcium (anti-VGCC P/Q-type) and potassium channels (VGKC) are shown. Four IP sera are weakly positive for anti-VGCC P/Q-type antibodies, and 2 STC sera strongly positive for anti-VGKC (arrowed). Dotted line: mean + 3SD; HC: Healthy controls; LEMS: Lambert-Eaton myasthenic syndrome; STC: Slow transit constipation; IP: Intestinal pseudo-obstruction; IMC: Idiopathic megacolon; NMT: Neuromyotonia.

Genomic investigations

Recent history has witnessed a colossal expansion in data regarding the human genome in health and disease. In keeping with this, several studies have demonstrated molecular genetic changes that accompany, and in some instances, contribute to various forms of intestinal dysmotility. Whilst offering interesting research insights, few presently have great value in clinical practice, and these are in the most part limited

to quite characteristic clinical syndromes. Most utilised are candidate single gene approaches, and these have been applied to screening for RET mutations in patients with Hirschsprung disease[43] or suspected multiple endocrine neoplasia (MEN) 2 syndromes[44], and thymidine phosphorylase mutation analysis in patients with MNGIE[42]. A variety of tests may also be appropriate in patients in which GI dysmotility may accompany other systemic diseases such as muscular dystrophy, cystic fibrosis and neurofibromatosis. In most cases, the information delivered is used to guide genetic counselling, and prognosis rather than influence diagnosis (except prenatally) or treatment (except in MEN where prophylactic surgery may be required to prevent subsequent neoplasia[45]).

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GUIDELINES CLINICAL PRACTICE

Asbjørn Mohr Drewes, Professor, MD, PhD, DMSc, *Series Editor*

Mucosal blood flow measurements using laser Doppler perfusion monitoring

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Abstract

Perfusion of individual tissues is a basic physiological process that is necessary to sustain oxygenation and nutrition at a cellular level. Ischemia, or the insufficiency of perfusion, is a common mechanism for tissue death or degeneration, and at a lower threshold, a mechanism for the generation of sensory signalling including pain. It is of considerable interest to study perfusion of peripheral abdominal tissues in a variety of circumstances. Microvascular disease of the abdominal organs has been implicated in the pathogenesis of a variety of disorders, including peptic ulcer disease, inflammatory bowel disease and chest pain. The basic principle of laser Doppler perfusion monitoring (LDPM) is to analyze changes in the spectrum of light reflected from tissues as a response to a beam of monochromatic laser light emitted. It reflects the total local microcirculatory blood perfusion, including perfusion in capillaries, arterioles, venules and shunts. During the last 20-25 years, numerous studies have been performed in different parts of the gastrointestinal (GI) tract using LDPM. In recent years we have developed a multi-modal catheter device which includes a laser Doppler probe, with the intent primarily to investigate patients suffering from functional chest pain of presumed oesophageal origin. Preliminary studies show the feasibility of incorporating LDPM into such catheters for performing physiological studies in the GI tract. LDPM has emerged as a research and clinical tool in preference to other methods; but, it is important to be aware of its limitations and account for them when reporting results.

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Key words: Laser Doppler perfusion monitoring; Gastrointestinal tract; Mucosal blood flow; Perfusion; Chest pain

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INTRODUCTION

Circulation and perfusion of individual tissues is a basic physiological process that is necessary to sustain oxygenation and nutrition at a cellular level. Ischemia, or the insufficiency of perfusion, is a common mechanism for tissue death or degeneration, and at a lower threshold, a mechanism for the generation of sensory signals including pain. Ischemia is a common cause of pain from the myocardium in coronary heart disease, ranging from reversible changes in angina pectoris to acute myocardial infarction with its multitude of complications. Myocardial ischemia is usually due to stenoses of the larger epicardial arteries; but, microvascular changes, such as those commonly seen in patients with diabetes mellitus, can cause ischemia and biochemical changes triggering pain signalling from the tissues. Ischemia can similarly cause pain from abdominal organs, including the intestines, when stenoses of the proximal mesenteric arteries limit perfusion in the distal vascular bed. It is also possible that abnormalities in smaller vessels, and in their regulation of blood flow may

cause similar biochemical changes in the intestinal wall and be an integral part of the pathogenesis of disease[1].

It is of considerable interest to study perfusion of peripheral abdominal tissues in a variety of circumstances. When studying the pathogenesis of various diseases, measurements of perfusion of the tissues affected may be essential to assess the relative contribution of ischemia to disease pathogenesis. Furthermore, in the surgical treatment of disease, assessment of perfusion may be important for assuring that anastomoses are established in well perfused segments of the gut. The beneficial or adverse effects of drug therapy might also be evaluated by monitoring perfusion of a segment of the gut.

MICROCIRCULATION IN THE GASTROINTESTINAL TRACT

Our knowledge of the peculiarities of the vascular bed of the gastrointestinal (GI) tract is still limited. There is considerable inter-individual variation in the anatomy of larger vessels, and the extent of collateral circulation, which may also explain differences in susceptibility to local ischemia. Perfusion is dependent on the arterial supply from the celiac, superior mesenteric and inferior mesenteric arteries. The watershed areas between these major arteries are likely to suffer from ischemia during acute or chronic arterial insufficiency.

The tissue volume occupied by moving blood cells is small; the average density of capillaries is about 50 capillaries per mm^2 of mucosal area, and on average 20% of capillaries are open under resting conditions, perfusion being mainly regulated by the opening and closing of precapillary sphincters. This autoregulation of perfusion is well established in several studies, including studies employing laser Doppler perfusion monitoring (LDPM) and is highly dependent on endothelial cell function[2].

MICROCIRCULATION AND THE SPECTRUM OF GASTROINTESTINAL DISEASE

Microvascular disease of the abdominal organs has been implicated in the pathogenesis of a variety of disorders, including peptic ulcer disease and inflammatory bowel disease (including both ulcerative colitis and Crohn's disease of the intestines). It has been suggested that apart from immunological and bacterial effects on the intestinal wall, changes in the microvasculature are essential for developing such key elements as mononuclear cell infiltration and fibrosis. Typically, the early stages of colitis show increased perfusion, whereas the later chronic stages of this disease show hypoperfusion of the mucosa. This was first shown in various animal models of acute inflammatory bowel disease (IBD), but also convincingly in patients with chronic disease with fibrosis[2]. Importantly, it has been found that the capacity for vasodilatation is decreased in chronic IBD, suggesting a mechanism for ischemia and pain[3]. It has been argued that in patients with Crohn's disease, chronic vascular changes may result in areas of microinfarction in the gut wall, leading to granulomatous inflammation and fibrosis[4].

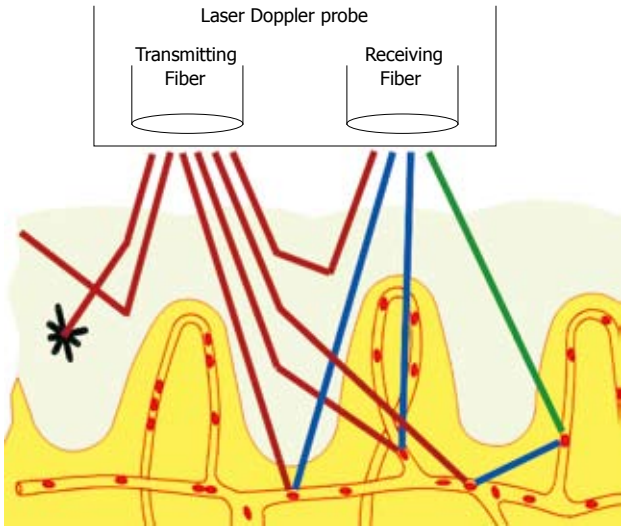

Figure 1 A schematic depiction of laser Doppler perfusion monitoring showing the probe with its emitting fibre bundle which applies monochromatic laser light to the tissue, and its receiving fibre bundle which returns reflected light for analysis. The light that has undergone a doppler shift due to moving blood cells in the tissues reflects the microcirculatory perfusion at a given time. Reproduced by permission of Perimed AB.

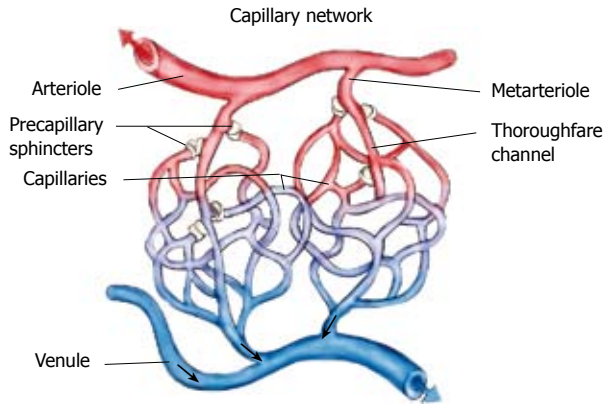

Figure 2 The capillary network showing also precapillary sphincters which regulate perfusion locally in response to metabolic needs, and shunts which participate in thermoregulation.

LASER DOPPLER PERFUSION MONITORING (LDPM)

The basic principle of laser Doppler perfusion monitoring (LDPM; laser Doppler velocimetry, or laser Doppler flowmetry) is to analyse changes in the spectrum of light reflected from living tissues as a response to a beam of monochromatic laser light emitted (Figure 1). LDPM reflects the total local microcirculatory blood perfusion including perfusion in the capillaries (nutritive flow), arterioles (thermoregulatory flow - such as in the skin), venules and shunts (Figure 2).

One of the earliest papers concerning this issue was a report by Stern *et al*[5] in 1975. They performed an ex-

periment to determine the feasibility of the method of coherent light scattering, in their case from a fingertip. They were able to demonstrate rapid microvascular reflexes which no other method was able to demonstrate at that time.

When a beam of light, carried by the fibre-optic probe, enters the tissues and hits moving blood cells in a random order, it undergoes changes in wavelength - a Doppler shift[6] - while the wavelength of light hitting static tissue structures is unchanged. The magnitude and frequency distribution of these changes in wavelength are directly related to the number of moving blood cells, but relatively unrelated to their direction of movement.

The tissue volume occupied by moving blood cells is generally small; the average capillary density is about 50 capillaries per mm^2 mucosal area, and most photons do not undergo a frequency shift, but are backscattered or absorbed[7]. The backscattered and Doppler broadened (extended) light carries information about the speed and concentration of blood cells traversing the scattering volume[8].

The quantity that is measured in LDPM is generally referred to as perfusion, and expressed in Perfusion Units (PU) which are arbitrarily chosen. In general it is not possible to change PU values into blood flow expressed as mL/min per g tissue; but, it can be done in specific preparations when calibration can be done. Perfusion is defined as the product of local velocity and concentration of blood cells[8]. Speed refers only to the magnitude (mm/s) of the velocity vector, and even though the majority (99%) of blood cells in the undisturbed microcirculation are red cells, LDPM does not selectively measure red cells.

Penetration into the tissue explored depends on the wavelength of the emitted light, and is regulated by differences in fibre diameter/separation. Penetration depth is also influenced, to a great extent, by factors such as structure and density of the capillary bed. The measuring depth is often defined as the depth below the tissue to which approximately 2/3 of the surface light penetrates, and returns back to the tissue surface. A typical probe today is designed using a solid-state laser with a wavelength of 780 nm, one transmitting and one receiving fibre and a fibre separation of 0.25 mm. This could lead to a sample depth of about 0.5-1.0 mm, and the sample volume could be estimated to 1 mm^3. This rather shallow measuring depth was the conclusion of several different studies published in the eighties[9-13]; but, other studies executed during the same period suggested that LDPM had a capacity for transmural measuring in the GI tract[14-17]. During the nineties a consensus was reached that LDPM monitors the microcirculation only in the mucosa and the upper submucosa of the GI tract.

Calibration generally has several purposes: to check the stability of the instrument; to establish the linearity of the instrument's response to blood flow; to establish a relationship between different instruments; and to relate the reading of the instrument to true perfusion, if possible. A gold standard for calibration of LDPM does not exist, and because the optical properties and distribution of blood vessels in the tissue are heterogeneous it is not realistic to calibrate the instrument to measure absolute blood flow. Therefore, the manufacturers of these instruments have provided a more simple calibration protocol, based on a two-point calibration, which makes it easy to calibrate the probes in a clinical or experimental situation. The motility standard is an aqueous suspension of polystyrene microspheres in Brownian motion. The method has some major shortcomings[8] regarding its dynamic properties, and the suspension induces Doppler shifts which give rise to a homodyne measurement. However, in living tissues, the opposite situation is seen and a heterodyne spectrum is produced because the majority of photons do not undergo a Doppler shift.

During the early years of LDPM, the method was validated against well established methods for measuring blood flow, such as the electromagnetic method. However, this method clearly measures total blood flow, not just blood flow in the microcirculation[9]. Later validation was performed using alternative methods known to selectively measure perfusion of the mucosal or muscularis layers i.e. local isotope washout[12], radioactive labelled microspheres[11], and H_2-clearance[11,13]. Generally, it is not easy to evaluate these validation studies because the single point laser Doppler probe is measuring from a different and much smaller tissue volume. However, carefully executed experiments performed on preparations of canine stomach and intestinal wall showed excellent linear correlation between the LDPM signal obtained and total blood flow measured by the electromagnetic technique[9,10,14,15,18]. One of these studies was also the first one to show that the gastric mucosa can autoregulate its blood flow, independent of other layers of the wall[10].

LDPM has emerged as a research and clinical tool in the absence of other methods, because it is a continuous, non-invasive and real time method for measuring microvascular blood flow, and it is also sensitive for detecting rapid changes in perfusion in the capillary circulation. LDPM is easily used in the clinical setting; but, to do so, one must be aware of its limitations. It is very important to ensure that the normal action and physiological responses of the microcirculation are not ignored when using this method. To get an optimal result there are both environmental and physical factors to take into consideration. These should be limited or accounted for when doing an investigation, in order to obtain reproducible data. It is also important to realise that it is impossible to say what the exact blood flow for any tissue is, and to remember that the optical properties and microvascular architecture cannot be determined in advance.

Physiological factors to be considered are temperature (thermoregulation has a significant effect on the microcirculation), the position and motion of the probe relative to the tissue surface, anatomical site and mental stress. Food and drugs also have effects on the microcirculation.

Technical limitations such as motion artifacts, multiple sequential Doppler shifts, variations in the specification of instruments from different manufacturers, lack

of exact knowledge of the depth of measurements, the instrument zero and/or biological zero[19] all have to be taken into consideration when analysing an investigation. There are several review articles published in recent literature describing these phenomena in more detail[8,20,21].

The laser Doppler probe is a sensitive motion detector, and many extraneous sources of noise e.g. respiratory movements cause mechanical vibrations in the same frequency range as the laser Doppler shifts produced by moving cells in the tissues (mucosa). Muscle fasciculation, vasomotion, respiration or any tissue movement relative to the laser Doppler probe may add noise to the laser Doppler signal. The GI organs are inherently motile, and motility-induced artefacts always occur during LDPM. One could argue that it is just noise which is recorded from the GI tract; but, Kiel *et al*[10] showed that this is not the case, and that true, perfusion can be measured from the GI tract.

Currently available instruments for LDPM generally also measure and display total backscattered light, of which Doppler shifted light makes up just a small fraction. The unit of measurement of backscattered light is EV, whereas that of Doppler shifted light is mEV. The significance of total backscattered light is that when this is detected as stable; it is an indication of minimal motion artifacts between probe and tissue. One can argue that only when backscatter is stable can we assume that LDPM actually measures perfusion in the adjacent tissues and is not simply dominated by artefacts.

THE APPLICATION OF LASER DOPPLER PERFUSION MONITORING TO STUDY DISEASE PROCESSES IN GASTROINTESTINAL TISSUES

During the last 20-25 years numerous studies have been done in different parts of the GI tract using LDPM. The majority of studies have been done on animals or humans during anaesthesia or surgery. This gives much better control of factors which potentially might influence the measurements. In a fully awake human, it is much more complicated to do LDPM, especially in the upper GI tract. A survey of the literature indicates that research employing LDPM has focussed on a limited number of questions, primarily those evaluating the influence of drugs or surgical procedures on mucosal perfusion, especially in the upper GI tract[22] or cardiovascular system[23], and the influence of septic shock[24], portal hypertensive gastropathy[25,26], or hepatic cirrhosis[27,28]. LDPM has certainly been used in some other clinical settings, but less systematically.

During recent years, we have been working with a multi-modal device (Figure 3) incorporating a laser Doppler probe, developing this device primarily in order to investigate patients suffering from functional chest pain of presumed oesophageal origin[29], an illness which is incompletely understood. Distending a bag in the oesophageal body typically reproduces the painful sensation in such patients but also elicits pain in a subset of healthy subjects[30-33]. The exact mechanism is unknown. We hypothesised that chest pain of presumed oesophageal origin could be due to a mechanical or an ischemic mechanism, leading to excitation of afferent nerves in the oesophageal wall.

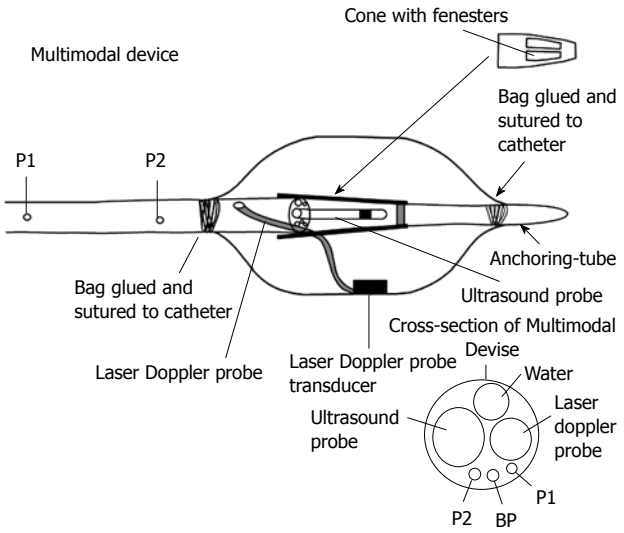

Figure 3 The multi-lumen PVC catheter (Outer Diameter = 6.0 mm) and a distal bag for acoustic coupling and symptom provocation. A water perfused manometric system measures pressures inside the bag (BP) and proximal to the bag at locations P1 and P2. The end of the multi-lumen catheter was attached to a fenestrated cone of polyethylene. The distal end of the cone was attached to a smaller end mounted catheter (anchoring-tube) for distal attachment to the bag. A 20 MHz ultrasound probe was placed in the centre of the bag and the transducer of the laser Doppler probe (780 nm) was fixed with double-sided tape to the inner surface of the bag. Modified from Hoff *et al*[34], 2006.

The multimodal catheter concept in gastroenterology was introduced in 2002 by Drewes and coworkers[32] who integrated technology for inducing electrical, mechanical, cold and heat stimuli into the same catheter device. We have developed the concept and technology further to include real time imaging with ultrasonography and LDPM[34]. As summarized in Figure 3, the device has a specially designed multi-luminal catheter as the central core, and a bag attached at its distal end. Inside the bag, as well as a sensor for measuring bag pressure, there is a radial 20 MHz miniature ultrasound probe (UM-3R, Olympus Corp, Tokyo, Japan) and a laser Doppler probe (LDP-415-253 (Perimed AB, Stockholm, Sweden). Its small size (10 mm × 6 mm × 4.5 mm, fibre diameter 140 μm, separation 250 μm, wavelength 780 nm) has facilitated its inclusion in the device. This is connected to a PF 5001 main unit with a PF 5010 LDPM Unit (Perimed AB).

In tests, high quality signals from manometry, LDPM and endosongraphy were obtained. The LDPM signal decreased moderately during bag distensions. Contractions characterized by high amplitudes and long duration were associated with a decrease in mucosal perfusion; but, minor fluctuations were also observed without contractile activity. During injection of 20 mg butylscopolamine bromide, fewer contractions were recorded and the LDPM signal fluctuated less.

LDPM can be obtained up to a depth of 1 mm with the type of equipment selected for our studies[8]. Hence, presumably at all degrees of bag distension, signals will originate primarily from the mucosa. Animal experiments have demonstrated residual compressive stresses in the mucosa-submucosa and tensile stresses in the muscle layers. This indicates more evenly distributed stress and strain throughout the oesophageal wall as also demonstrated in a study of the multilayered composite oesophagus by Liao and co-workers[35]. It is, therefore, likely that changes in perfusion throughout the wall are also evenly distributed. No current method can provide reliable flow data from the entire human oesophageal wall, and LDPM seems to be the best available choice, particularly for the multimodal device.

This is, to our knowledge, the first time LDPM has been included in a multimodal device for measuring perfusion in the oesophageal wall. Preliminary studies show the feasibility of the method; but, obviously the present material does not allow firm conclusions about whether GI pain is primarily of mechanical or ischemic origin. Future studies to look into ischemic- or strain-dependent pain mechanisms, may need to employ advanced distension protocols such as strain softening protocols.

CONCLUSION

Laser Doppler perfusion monitoring has emerged as a research and clinical tool in preference to other methods because it is non-invasive, and yields continuous and real-time measurements of microvascular blood flow. Furthermore, it is sensitive to rapid changes in perfusion in the capillary circulation. LDPM is easily used in the clinical setting; but, users have to be aware of its limitations and account for them when reporting results. LDPM can be included in multimodal devices, and we have demonstrated that simultaneous measurements of pressure, perfusion and ultrasound can be obtained from the oesophagus, when combined with bag distension. The quality of the data indicates that new insights can be obtained from studies in healthy volunteers and patients with functional chest pain. There are still some major challenges to face due to the fact that the method is highly motion-sensitive, and we cannot give the exact depth location from where perfusion measurements are obtained.

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BRIEF ARTICLES

Prevalence of *vacA*, *cagA* and *babA2* genes in Cuban *Helicobacter pylori* isolates

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Abstract

AIM: To investigate the prevalence of vacuolating cytotoxin (*vacA)*, cytotoxin associated gene A (*cagA)* and blood adhesion binding antigen (*babA2)* genotypes of *Helicobacter pylori* (*H pylori*) isolates from Cuban dyspeptic patients.

METHODS: DNA was extracted from *H pylori*-positive cultures taken from 130 dyspeptic patients. Genotyping was performed by PCR, using specific primers for *vacA* (*s1*, *s2*, *m1*, *m2*), *cagA* and *babA2* genes. Endoscopic observations and histological examinations were used to determine patient pathologies.

RESULTS: *vacA* alleles *s1*, *s2*, *m1* and *m2* were detected in 96 (73.8%), 34 (26.2%), 75 (57.7%) and 52 isolates (40%), respectively, while the *cagA* gene was detected in 95 isolates (73.2%). One hundred and seven isolates (82.3%) were *babA2*-positive. A significant correlation was observed between *vacAs1m1* and *cagA* and between *vacAs1m1* and *babA2* genotypes ($P < 0.001$ and $P < 0.05$, respectively) and between *babA2* genotype and *cagA* status ($P < 0.05$); but, no correlation was observed between *vacAs1* and *babA2* genotypes. Eighty five (65.4%) and 73 (56.2%) strains were type 1 (*vacAs1-cagA*-positive) and "triple-positive" (*vacAs1-cagA-babA2*-positive), respectively, and their presence was significantly associated with duodenal ulcer ($P < 0.01$ and $P < 0.001$, respectively).

CONCLUSION: The distribution of the main virulence factors in the Cuban strains in this study resembled that of the Western-type strains, and the more virulent *H pylori* isolates were significantly associated with duodenal ulcer, ulcer disease being the worst pathology observed in the group studied.

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Key words: Cuban dyspeptic patients; *Helicobacter pylori*; *vacA*; *cagA* and *babA*

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INTRODUCTION

Helicobacter pylori (H pylori), a spiral-shaped microaerophilic bacterium infects more than 50% of the world's population, the rate of infection being higher in developing countries[1]. *H pylori* is a major etiological agent in several gastroduodenal diseases, such as functional dyspepsia, peptic ulcer disease, gastric cancer and mucosa-associated lymphoid tissue lymphoma. The clinical outcome following infection with this pathogen has been related to environmental conditions, host

immunological factors and microorganism virulence[2].

Vacuolating cytotoxin (VacA), cytotoxin associated gene A (*cagA*), and blood adhesion binding antigen (*babA*) are the most commonly studied virulence markers of *H pylori*. However, there are other bacterial proteins with pathogenic potential, such as sialic acid-binding adhesin (SabA), outer inflammatory protein (*oipA*), and duodenal ulcer promoting gene (*dupA*); but, the influence of these proteins on *H pylori* pathogenesis is still under study[3].

The VacA protein induces vacuolation and apoptotic processes in epithelial cells, as well as immunosuppressive actions in immunological cells[4]. The *vacA* gene comprises two main regions: the signal zone (s1 or s2) and the middle region (m1 or m2)[5]. The *vacA s1m1* allelic combination exhibits the highest activity, while *s2m2* and the rare *s2m1* combinations are non-toxic[5]. Recently, a new polymorphic region in the *vacA* gene called the intermediate region (*i*) has been discovered and its *i1* active allele seems to be a better predictor of gastric cancer than the *s1* or *m1* allele[6].

Hydrophilic protein CagA contains the so-called EPIYA motifs[7], which interact with several eukaryotic proteins, promoting changes in the signal transduction pathway, cytoskeletal plasticity and IL-8 secretion in epithelial cells[8]. CagA-positive *H pylori* isolates are associated with a higher rate of gastric inflammation and damage, when compared with CagA-negative strains[8,9]. The *cagA* gene is located at the end of the cag pathogenecity island, a system that introduces CagA and a peptidoglycan into epithelial cells[10]. Several epidemiological studies have shown the correlation between *cagA*-positive strains and a higher risk of developing peptic ulceration, gastric atrophy and gastric cancer[8,9].

The blood group binding antigen mediates adherence of *H pylori* to human gastric epithelium[11]. This antigen is encoded by the polymorphic gene called *babA2*, while allele *babA1* is non-functional[11]. Some studies have suggested that BabA plays a crucial role in the development of severe functional dyspepsia, peptic ulcer and gastric adenocarcinoma[12,13]. Furthermore, the combined presence of *vacAs1* and *cagA* genotypes (type 1 strains) or even the "triple-positive" strains (*vacAs1*, *cagA* and *babA2*), has shown a higher correlation with the appearance of peptic ulcer, intestinal metaplasia and gastric cancer[14].

The clinical outcome of this bacterial infection seems to be influenced by the distribution of the above-mentioned pathogenic factors in *H pylori* strains[15]; but, complete genotyping of Cuban *H pylori* strains has never been carried out. Therefore, the aim of this study was to determine the frequency of the main virulence factor genes in Cuban *H pylori* isolates and establish their associations with the clinical outcome.

MATERIALS AND METHODS

Patients

H pylori isolates were obtained from 130 consecutive *H pylori*-positive patients (77 male and 53 female) with a mean age of 49.1 years (range, 18 to 88) who underwent routine endoscopy due to dyspeptic complaints at CIMEQ Hospital, Havana, Cuba. Endoscopic observation and histological confirmations were used to determine patient pathologies. This study was approved by the ethics committee at CIMEQ Hospital. All patients provided informed consent to participate in the study.

Microorganism culture

Antrum gastric biopsy specimens obtained from all patients were homogenized, inoculated into Columbia agar base plates with 7% human blood and SR0147E selective supplement (Oxoid, England, UK), and grown under microaerophilic conditions at 37℃ for 5 to 8 d. All *H pylori* isolates were positive for oxidase, catalase and urease. The reference strain J99[16] was kindly provided by Professor Francis Megraud from Pellegrin Hospital, Bordeaux, France.

DNA extraction and cagA, vacA and babA2 genotyping

Genomic DNA was extracted by CTAB methodology with phenol/chloroform and isopropanol precipitation as previously described[17]. Purified DNAs were stored at -20℃ until use. In all cases, PCR amplification was carried out in a 25 μL reaction mixture containing 2.5 μL 10X PCR buffer (Roche, Germany), 0.2 mmol/L of each deoxynucleotide triphosphate, 0.6 mM sense and antisense primers, 4 mmol/L magnesium chloride, 1.25 U Taq DNA polymerase (CIGB, Cuba) and 100 ng genomic DNA. The PCR had an initial step at 94℃ for 1 min, followed by 40 cycles at 94℃ for 1 min, 60℃ for 1 min and 72℃ for 1 min, and a final extension at 72℃ for 5 min, using a Master Cycler apparatus (Eppendorf, Germany).

The primers used and their details are shown in Table 1. Primers to the *glmM* gene of *H pylori* were used to control DNA integrity and specificity. PCR products were analyzed on 1.5% agarose gel electrophoresis with ethidium bromide. Images were taken through the Gene Genius system (Syngene, England, UK).

Statistical analysis

Differences among groups were tested using the χ^2 test. P values < 0.05 were considered to be significant. The statistic software, version 8 for Windows, was used for statistical analysis.

RESULTS

Detection of H pylori genotypes

H pylori was successfully cultured from 130 Cuban dyspeptic patients. DNA integrity and specificity was confirmed by *glmM* PCR, which rendered the expected 417 bp band from all isolates (data not shown). PCR product sizes of *vacA s* and *m* alleles were used to differentiate them in agarose gels (Figure 1, panel A). The most virulent *vacAs1* allele was predominantly present in Cuban *H pylori* isolates (Table 2), and

Table 1 Primer used for PCR genotyping of Cuban *H pylori* strains

Primer	Sequence (5'-3')	AT ℃	Size (bp)	Ref.
glmMF	CCCTCACGCCATCAGTCCCAAAAA	60	417	[18]
glmMR	AAGAAGTCAAAAACGCCCCAAAAC			
cagF1	GATAACAGGCAAGCTTTTGA	60	349	[7]
cagB1	CTGCAAAAGATTGTTTGGCAGA			
vacAsF	ATGGAAATACAACAAACACAC	52	*s1*-259/*s2*-286	[20]
vacAsR	CTGCTTGAATGCGCCAAAC			
vacAmF	CAATCTGTCCAATCAAGCGAG	56	*m1*-567/*m2*-642	[20]
vacAmR	GCGTCAAAATAATTCCAAGG			
bab7-F	CCAAACGAAACAAAAAGCGT	60	271	[21]
bab7-R	GCTTGTGTAAAAGCCGTCGT			
babA2F[1]	AATCCAAAAAGGAGAAAAAGTATGAAA	60	832	[13]
babA2R	TGTTAGTGATTTCGGTGTAGGACA			
babA2R607[2]	GTTTTCTTTGAGCGCGGGTAAGC	60	607	[14]

[1]Forward primer used with primer babA2R or babAR607 to amplify *babA2* gene; [2]Five nucleotides (GTTTT) were added to the original primer designed by Zambon *et al*[14] to increase specificity.

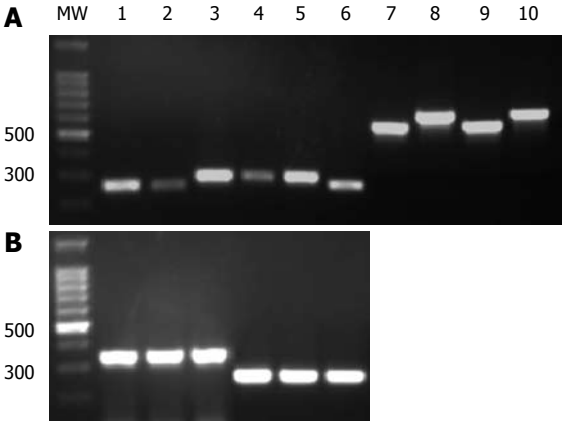

Figure 1 Genotyping of main virulence factor genes in Cuban *H pylori* isolates. The images shown are from a representative gel electrophoresis of two independent PCR amplification products of *vacA* (*s1*, *s2*, *m1*, *m2*), *cagA* and *babA2* genes from Cuban isolates and J99 control strain. A: Lanes 1 and 7, reference strain J99 (*vacAs1* and *m1* alleles, respectively); Lanes 2 and 6, *vacAs1* strains; Lanes 3-5, *vacAs2* strains; Lanes 8 and 10 *vacAm2* strains; Lane 9, *vacAm1* strain. B: Lanes 1 and 4, J99 strain (*cagA* and *babA2* gene, respectively); Lanes 2 and 3, *cagA*-positive strains; Lanes 5 and 6 *babA2*-positive strains. MW: 100 bp DNA Ladder (Promega, USA).

was visualized as a band of 259 bp on agarose gel electrophoresis (Figure 1, panel A), whereas 26.2% of isolates had the *vacAs2* genotype (Table 2). The middle region of the *vacA* gene was detected in only 127 of the 130 isolates, *m1* and *m2* genotypes were more equally distributed than s genotypes (Table 2). On the other hand, *s1m1* and *s2m2* genotypes were the most common allelic combinations of the *vacA* gene among Cuban isolates, and only one strain harbored the *s2m1* genotype (Table 2).

Amplification of the *cagA* gene was visualized as a band of 349 bp (Figure 1, panel B) and was present in 73.2% of the strains (Table 2). When primers babA7F/babA7R (Table 1) were used to amplify the *babA2* gene, over 80% of the Cuban strains carried this gene (Table 2). In contrast, a low prevalence of *babA2* genotype was observed among the Cuban isolates when using primers babA2F/babA2R and babA2F/babA2R607 (Table 1).

Table 2 Correlation between *vacA* alleles and *cagA* and *babA2* genotypes in 130 Cuban *H pylori* isolates

vacA	*s1m1*	*s1m2*	*s2m2*	*s2m1*	*s1m-*	Total (%)
cagA+	70	14	8	1	2	95 (73.2)
cagA-	4	5	25	0	1	35 (26.8)
babA2+	67	14	24	1	1	107 (82.3)
babA2-	7	5	9	0	2	23 (17.7)
Total (%)	74 (56.9)	19 (14.6)	33 (35.4)	1 (0.8)	3 (2.3)	130

Table 3 Correlation between virulence factor genotypes and disease outcome

Genotypes	Pathologies		
	FD *n* = 51 (%)	GU *n* = 33 (%)	DU *n* = 46 (%)
vacAs1m1	28 (54.9)	16 (48.5)	30 (65.2)
s1m2	5 (9.7)	9 (27.3)	5 (10.9)
s2m2	16 (31.4)	6 (18.2)	11 (23.9)
s1m-	1 (2)	2 (6)	-
s2m1	1 (2)	-	-
cagA+	36 (70.6)	19 (57.6)	40 (87)
babA2+	36 (70.6)	28 (84.8)	43 (93.5)[b]
Type 1	29 (56.9)	16 (48.5)	40 (87)[d]
Triple-positive	24 (47.1)	12 (36.4)	37 (80.4)[f]

FD: Functional dyspepsia; GU: Gastric Ulcer; DU: Duodenal Ulcer; *P* values were calculated with the χ^2 test; [b, d, f]Statistically significant differences (*P* values < 0.01).

Combinations of vacA, cagA and babA2 genotypes

On examining the association of the main virulence genes in each strain, a statistically significant correlation was observed between *s1m1* genotype and *cagA* status ($P = 0.00001$), between *s1m1* and *babA2* genotypes ($P = 0.047$), and between *cagA* and *babA2* genotypes ($P = 0.049$). A significant association was also observed between *vacAm1* allele and *cagA* status or *babA2* genotype ($P = 0.00001$ and $P = 0.035$, respectively), while most *s2m2* strains carried a *cagA*-negative genotype (Table 2). However, no correlation was observed between *vacAs1* and *babA2* genotypes ($P = 0.12$). Additionally, 85 isolates were classified as type 1 strains and 73 were triple-positive strains (Table 3).

Table 4 Worldwide distribution of main H. pylori virulence factors

World Area	*vacA* alleles prevalence (%)				References	*cagA* prevalence (%)	*babA2* prevalence (%)
	s1	*s2*	*m1*	*m2*		*cagA* +	*babA2* +
Europe	48-89	11-51	37	63	[14,23,26,27]	66-73[14,23,26,27]	34-72[13,14,34]
America	57-68	16-48	37-44	29-63	[19,20,24]	57-75[7,19,24]	46-69[24,32]
East Asia	100	0	41-94	5-55	[12,25,28]	90-100[12,25,28,35]	80-100[12,21,35]

Relationship between genotypes and gastric diseases

Of the 130 *H pylori* infected patients studied, 39.2% were diagnosed with functional dyspepsia, 35.4% had a duodenal ulcer (DU) and 25.4% had a gastric ulcer (GU). Table 3 shows that the *vacAs1m1* genotype was detected at a higher frequency in isolates from patients with DU, and in strains obtained from patients with functional dyspepsia; but, the presence of this genotype did not correlate with the presence of duodenal or gastric ulcer (P = 0.21 and P = 0.4, respectively). On the other hand, the *vacA s1m1* genotype had a higher frequency in DU patients; but, no association was observed between *s1m1* or any other *vacA* genotype, and the presence of severe pathologies in this study (Table 3). GU patients exhibited the highest frequency of *s2m2* strains, followed by patients with functional dyspepsia (Table 3). No correlation was found between the *cagA* genotype and duodenal or gastric ulcer (P = 0.051 and P = 0.22, respectively); but, an association between *cagA*-positive strains and DU may be assumed as a clear tendency (Table 3). Meanwhile, the *babA2* genotype was significantly associated with DU (P = 0.004), but not with GU (P = 0.13). Type 1 and triple-positive strains (Table 3) were also associated with DU (P = 0.001 and P = 0.0007, respectively) but not with GU (P = 0.45 and P = 0.33, respectively).

DISCUSSION

Several studies have shown that the incidence and/or severity of gastroduodenal pathologies related to *H pylori* may vary between geographic areas. This phenomenon is partly due to a different distribution of pathogenic markers in circulating strains[15]. Several pathogenic factors of *H pylori* have been described and their association with the clinical outcome studied[19-21]. Distribution of the main virulence factors around the world is summarized in Table 4, showing the high variation between geographic areas. This is the first report to examine the three main *H pylori* virulence associated genes, *vacA*, *cagA* and *babA2* in Cuban isolates.

vacA alleles

The *vacA s1* and *s2* leader sequences are different in a small insert, totaling 27 bp, carried by the *vacAs2* allele[20], which has a reduced capacity to secrete VacA toxin[22]. According to our results, the most virulent *vacAs1* allele was predominant in Cuban *H pylori* isolates (Table 2), a finding which has also been observed in other studies of Western strains (Table 4)[23,24]. In the present study, the prevalence of *vacAm1* and *vacAm2* were similar compared to that of the *s1* and *s2* allele; meanwhile, the *s1m1* and *s2m2* genotypes were the most common allelic combinations of the *vacA* gene from Cuban isolates (Table 2), a finding reported in several studies from various countries[19,20]. Furthermore, the middle region of *vacA* was not detected in three isolates, while only one strain harbored the *s2m1* genotype. Genotyping of the *vacA* middle region failed in three strains, probably due to heterogeneity in the *vacA* gene, a finding described previously[12,24]. Additionally, only one strain harbored the *s2m1* genotype, the *vacA* allelic combination relating to lower incidence in several studies[23,24]. On the other hand, the *vacA s1m1* genotype was noted at a higher frequency in DU patients; but, no significant correlation was observed between *vacA* genotypes and the appearance of peptic ulcer disease, which is in agreement with previous reports[19,25].

cagA genotype

H pylori cagA-positive strains have been associated with more severe gastroduodenal diseases[8,14,15]. Here, 73.2% of the *H pylori* strains were *cagA*-positive, a prevalence similar to that reported in many studies from Western countries (e.g. USA, 60%[7]; Spain, 66%[26]; and England, 68%[27]) but lower than that reported in some East Asian studies, which encountered over 90% of *cagA*-positive isolates (Table 4)[25,28]. In addition, a highly significant correlation was observed between *cagA* status and *vacAs1* and *vacAs1m1* genotypes (Table 2), which is commonly linked to an increase in *H pylori* virulence[13,14,29,30]. An association was also observed between the presence of the *cagA* gene and the *babA2*-positive genotype, due to the fact that most *cagA*-positive isolates carried the *babA2* allele. Our data support the relationship between *cagA* and *babA2* genes found in previous reports, which could be caused by selective pressure[13,14], although other authors, such as Mattar *et al*[24], did not find any correlation between these virulence factors in the isolates investigated. On the other hand, previous studies have shown a high association between the *cagA*-positive genotype and the appearance of DU[31,32]. In this study, however, no correlation was observed between *cagA* status and DU. Moreover, a high frequency of *cagA*-positive strains was observed in DU patients (Table 3), indicating that a statistical association could be reached by increasing the number of patients in future studies.

babA2 genotype

Adherence of *H pylori* to epithelial cells is a relevant step in the development of gastroduodenal pathologies. *BabA2* attaches *H pylori* to these cells, allowing delivery of VacA and CagA toxins near the gastric epithelium and therefore enhancing gastric tissue damage[3,11]. Here, Cuban *H pylori* isolates exhibited a high frequency (82.3%) of the *babA2* allele when the primers of Sheu *et al*[21] were used to amplify the gene. In contrast, a low prevalence of the *babA2* genotype was observed when the primers reported by Gerhard *et al*[13] and a variant of Zambon *et al*[14] were used, respectively (Table 2). Interestingly, these last two primers are located in a high polymorphic zone of the *babA* gene[33], which should lead to an underestimation of *babA2*-positive strains. Our results add new data to previous observations[24,34] that support the ineffectiveness of the Gerhard *et al*[13] primers to detect the *babA2* gene, and for the first time relate this to deficiencies in the primers used by Zambon *et al*[14]. Consequently, the low levels of *babA2* alleles reported in several previous studies[13,14,32] may be underestimated, due to the use of Gerhard primers[13]. However, the prevalence of the *babA2* gene was above 70% in Asian countries using the same primers[12,35], suggesting that underestimation due to allelic variation in the *babA* gene could have a variable impact in different geographic areas, as was previously suggested[34]. This study showed a high association between the presence of the *babA2* allele and DU disease (Table 3), which is in agreement with several reports which associate the presence of this gene with the appearance of severe gastric damage[13,14]. However, other studies have claimed no association between this genotype and more severe pathologies[24].

Combination of virulence genotypes

Of the 130 Cuban *H pylori* isolates, 65.4% and 56.2% were type 1 and triple-positive strains, respectively. Infection with these strains has been associated with a higher degree of inflammation and gastroduodenal lesions[14]. Similar percentages of both types of strains were found in this study and in a previous report[13], while Brazilian dyspeptic patients seem to have a lower rate (32.6%) of triple-positive strains[24]. Our data indicate that type 1 and triple-positive strains increase the risk of developing DU in Cuban dyspeptic patients, a finding consistent with other studies, in which these types of strains were mainly found in subjects with peptic ulcer disease[13], and in patients with intestinal metaplasia and gastric atrophy[14].

We hypothesize that the absence of a correlation between the virulence genes analyzed and the development of GU might be influenced by the small number of patients with this pathology in our study, although several other studies have not found any correlation between the presence of *H pylori* main virulence factor genes (alone or in combinations) and peptic ulcer disease[24,25].

It is interesting to note that despite the high rate of *H pylori* infection in Cuban dyspeptic patients[36-38], and the relatively high pathogenic potential of Cuban isolates found previously[37,38] and in the present study, a low incidence of gastric adenocarcinoma has been found in Cuban patients with dyspepsia[36-38]. This reflects a general tendency in the Cuban population towards low levels of gastric cancer; in fact, a gastric cancer death rate of 7.1/100 000 was observed in Cuba in 2007 (http://www.sld.cu/servicios/estadisticas/). Future studies are required to elucidate the above-mentioned investigative problem, including a full characterization of Cuban *H pylori* isolates.

In conclusion, this study has shown a relatively high prevalence of the main virulence factor genes in Cuban *H pylori* isolates, which is similar to that found in the Western-type strains. In addition, a significant association was found between the virulence genes in Cuban strains. Consequently, the presence of more virulent type 1 and triple-positive strains was relatively high in Cuban dyspeptic patients, and increased their risk of developing duodenal ulcer. On the other hand, more severe gastroduodenal pathologies, such as intestinal metaplasia, gastric atrophy and gastric cancer were not found in this study, or in other similar studies and which might merit further research.

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COMMENTS

Background

Cuba has a high incidence of *H pylori* infection. The presence and association of the main virulence factors VacA, CagA and BabA2 in *H pylori* strains influences the clinical outcome following infection with this pathogen. So far, no whole genotyping of Cuban *H pylori* strains has been carried out. This study addresses the frequency and association of the main virulence factor genes in *H pylori* isolates, and establishes their relationship to clinical outcome in a Cuban dyspeptic population.

Research frontiers

In a dyspeptic population in Cuba, the presence and association of the main virulence factor genes (*vacA*, *cagA* and *babA2*) in the infecting strains was significantly high, and their combined presence is a risk factor for duodenal ulcer (DU), but is not associated with gastric ulcer (GU). More severe pathologies, such as intestinal metaplasia, gastric atrophy and gastric cancer were not present in the group studied.

Innovations and breakthroughs

Studies of various populations have indicated an association between the presence of *vacA*, *cagA* and *babA2* genes in *H pylori* isolates and the appearance of more severe gastroduodenal pathologies. The distribution of these virulence markers in *H pylori* strains varies among populations. The present study showed a relatively high prevalence of the main virulence factor genes in Cuban *H pylori* isolates, similar to that found in the Western-type strains. In addition, the study demonstrated a significant association between the virulence genes in the strains studied, which was related to the risk of developing DU, but not GU in dyspeptic patients. Furthermore, despite the relatively high virulence potential of Cuban *H pylori* isolates, pathologies such as intestinal metaplasia, gastric atrophy and gastric cancer were not present in the dyspeptic population studied.

Applications

In developing countries with a high incidence of *H pylori* infection and dyspepsia, it is important to screen the isolates for main virulence factors. The information generated here may be used to develop a procedure to detect *H pylori* pathogenic factors in a given population from biopsy samples. Intervention may then be concentrated on subjects with a higher risk of severe pathologies.

Peer review

This study determined the prevalence of main virulence factor genes *vacA*, *cagA* and *babA2* in Cuban *H pylori* isolates and their association with gastroduodenal diseases.

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Role of bacterial and genetic factors in gastric cancer in Costa Rica

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Abstract

AIM: To evaluate several risk factors for gastric cancer (GC) in Costa Rican regions with contrasting GC incidence rate (GCIR).

METHODS: According to GCIR, 191 *Helicobacter pylori* (*H pylori*)-positive patients were classified into groups A (high GCIR, *n* = 101) and B (low GCIR, *n* = 90). Human DNA obtained from biopsy specimens was used in the determination of polymorphisms of the genes coding for interleukin (IL)-1β and IL-10 by PCR-RFLP, and IL-1RN by PCR. *H pylori* DNA extractions obtained from clinical isolates of 83 patients were used for PCR-based genotyping of *H pylori cagA*, *vacA* and *babA2*. Human DNA from gastric biopsies of 52 GC patients was utilized for comparative purposes.

RESULTS: Cytokine polymorphisms showed no association with GCIR variability. However, gastric atrophy, intestinal metaplasia and strains with different *vacA* genotypes in the same stomach (mixed strain infection) were more frequently found in group A than in group B, and *cagA* and *vacA s1b* were significantly associated with high GCIR (*P* = 0.026 and 0.041, respectively). IL-1β+3954_T/C (OR 2.1, 1.0-4.3), IL-1RN*2/L (OR 3.5, 1.7-7.3) and IL-10-592_C/A (OR 3.2, 1.5-6.8) were individually associated with GC, and a combination of these cytokine polymorphisms with *H pylori vacA s1b* and *m1* further increased the risk (OR 7.2, 1.4-36.4).

CONCLUSION: Although a proinflammatory cytokine genetic profile showed an increased risk for developing GC, the characteristics of *H pylori* infection, in particular the status of *cagA* and *vacA* genotype distribution seemed to play a major role in GCIR variability in Costa Rica.

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Key words: Costa Rica; Gastric cancer; *Helicobacter pylori*; Host genetic factors

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INTRODUCTION

Costa Rica has one of the highest age-adjusted incidence and mortality rates for gastric cancer[1]. In fact, this country reported the highest age-adjusted gastric cancer mortality rate in males and females over the period 1983-1997, out of a total of 30 countries, including Japan and Chile[2].

Costa Rica has regions with contrasting gastric cancer incidence rates (GCIR). Topographically, the central part of the country is predominantly composed of regions with high GCIR while coastal areas are largely characterized by low GCIR[3]. Population density varies according to geographic area. While in coastal regions the population density is around 30 persons per square km, in the central regions of San Jose and Cartago, it ranges from 140 to 270 persons per square km. Cultural, behavioral and dietary patterns are very similar throughout the country, regardless of population density[3]. The pre-

dominant ethnic group is the criollo, which has Spanish ancestry. In spite of these homogeneous patterns, the GCIR in Costa Rica shows a distinctive regional variation[4]. Several environmental factors such as the components of drinking water, soil and nutrients have been compared in contrasting GCIR regions; however, none of these factors was significantly associated with GCIR variation in the country[4,5].

The cause of gastric cancer is thought to be multifactorial. A higher incidence of gastric cancer in blood type A subjects than in those with other blood types was reported as early as the 1950's[6,7]. Several decades later, after the discovery of *Helicobacter pylori* (*H pylori*), which is a Gram-negative microaerobic bacterium that persistently colonizes the human gastric mucosa, it was reported that *H pylori*-positive subjects are believed to have a two- to three-fold increased risk of developing gastric cancer when compared with *H pylori*-negative subjects[8-11]. The risk is even higher in subjects infected with strains co-expressing the *H pylori cagA*, *vacA s1* and *babA2* genes[12-15]. Recently, cytokine gene polymorphisms of the host, IL-1β, IL-1RN and IL-10, in response to *H pylori* infection, have been associated with an increased risk for developing gastric cancer[16-21]. Moreover, it has been suggested that an interaction between a host's immunological defenses, environmental and *H pylori* virulence factors play a main role in the development of gastric cancer[22,23].

We previously reported that the presence of serum CagA antibody was found to be significantly higher in high GCIR regions than in low GCIR regions in Costa Rica, despite the fact that no significant difference was found in the prevalence of *H pylori* infection between the regions, suggesting that the *H pylori cagA* gene was associated with the development of severe gastric injury, glandular atrophy and cancer, which probably influenced the GCIR variability in the country[24]. However, further investigation is needed to demonstrate a significant association of *H pylori* and/or host factors with GCIR variability in Costa Rica.

The aim of this study was to evaluate whether host genetic factors such as interleukin (IL)-1β (-511 and +3954), IL-10 (-1082 and -592) and IL-1RN intron 2 variable number of tandem repeat (VNTR) polymorphisms in response to *H pylori* infection, and/or *H pylori cagA*, *vacA* and *babA2* genotype distribution could be associated with the GCIR variability present in Costa Rica.

MATERIALS AND METHODS

Study population

The patients in this study attended a digestive center in San Jose, Costa Rica. Out of 402 continuous dyspeptic patients who underwent upper endoscopy from January to July 2005 and from January to July 2006, a total of 191 *H pylori*-positive patients (80 males, 111 females; age range 23-76 years) were enrolled for the determination of cytokine gene polymorphisms in *IL-1β*, *IL-1RN* and *IL-10*. Clinical isolates successfully obtained from both antrum and corpus specimens of 83 patients were eventually utilized for the PCR-based genotyping of the *H pylori cagA*, *vacA* and *babA2* genes. Informed consent was obtained from each patient and the study was approved by the Ethics Committee of the institution.

In addition, gastric tissue specimens obtained from 52 consecutive *H pylori*-positive gastric cancer patients (GC group) who underwent surgical treatment at a hospital in Cartago, Costa Rica between February 2006 and March 2007, were utilized in this study to determine cytokine gene polymorphisms of the host, and were used for comparative purposes.

Based on a previous study[4], dyspeptic patients were classified into either high or low GCIR groups. Group A (high GCIR) was composed of patients belonging to regions with a GCIR in the range of 24.7-48.5/100 000 persons, while in group B (low GCIR) the incidence rates ranged from 9.8-19.9/100 000 persons. Patients belonging to regions with a GCIR of 20.0-24.6/100 000 persons were removed from the study to further distinguish group A from group B. Information on age, gender, place of origin, symptoms and medication was collected. Patients with a recent intake of proton pump inhibitors, antibiotics, non-steroidal anti-inflammatory drugs, or any drug that could alter the state of the gastric mucosa were excluded from this study. Likewise, patients with *H pylori* eradication or previous attempted eradication therapy, previous gastric surgery as well as patients with Asian ancestry were also excluded from the study.

Endoscopic and histological evaluations

Endoscopy was performed with Olympus Evis Excera 160 and 180 videoendoscopes (Olympus America Inc., San Jose, CA, USA). From each patient, five biopsies (two from the antrum, two from the corpus and one from the cisura angularis) were collected for histological examination. Two more biopsies (one from the antrum and one from the corpus) were also taken to obtain the isolates following bacterial culture.

The five biopsy samples from each of the 191 patients were conventionally fixed in 100 mL/L aqueous formaldehyde, and embedded in paraffin. Serial 3- to 4-μm sections were stained with hematoxylin and eosin for histological observation. Each biopsy specimen was evaluated independently by two experienced pathologists blinded to the endoscopic and laboratory examinations. All discrepant diagnoses were re-examined by both pathologists together in order to reach a final consensus diagnosis. All five biopsies were examined for the presence of glandular atrophy and intestinal metaplasia and were scored into one of four grades (0: none, 1: mild, 2: moderate and 3: marked) for both the antrum and the body of the stomach, according to the updated Sydney System of classification and grading of gastritis[25]. Gastric glandular atrophy was defined as the loss of gastric glands, and its replacement with fibrosis or metaplastic epithelium. Intestinal metaplasia was defined as the presence of foci where at least three neighboring gastric pits containing two or more goblet cells (in each pit) were visualized in any part of the stomach.

Table 1 PCR primers for amplification of *cagA*, *vacA* and *babA2* genes

Region	Primer	Nucleotide sequence	Reference
cagA	D008	5'-ATAATGCTAAATTAGACAACTTGAGCGA-3'	[28]
	R008	5'-TTAGAATAATCAACAAACATCACGCCAT-3'	
	cagAFnz3	5'-AAAAGCGACCTTGAAAATTCC-3'	[29]
	cagA-seqR1	5'-TAGCATAATTGTCCAATTTCGC-3'	
vacA s1	VA1-F	5'-ATGGAAATACAACAAACACAC-3'	[30]
	VA1-R	5'-CTGCTTGAATGCGCCAAAC-3'	
vacA s1a	S1A-F[1]	5'-TCTYGCTTTAGTAGGAGC-3'	[30]
vacA s1b	SS3-F[1]	5'-AGCGCCATACCGCAAGAG-3'	[30]
vacA s1c	S1C-F[1]	5'-CTYGCTTTAGTRGGGYTA-3'	[13]
vacA s2	VA1-F[1]	5'-ATGGAAATACAACAAACACAC-3'	[30]
vacA m1	VA3-F	5'-GGTCAAAATGCGGTCATGG-3'	[30]
	VA3-R	5'-CCATTGGTACCTGTAGAAAC-3'	
vacA m2	VA4-F	5'-GGAGCCCCAGGAAACATTG-3'	[30]
	VA4-R	5'-CATAACTAGCGCCTTGCAC-3'	
babA2	babA-F	5'-AATCCAAAAAGGAGAAAAAGTATGAAA-3'	[31]
	babA-R	5'-TGTTAG TGATTTCGGTGTAGGACA-3'	
	babA2-Fnc1	5'-GAAAAAACATGAAAAAACACATCCTTTCAT-3'	
This study			
	babA2-Rmn2	5'-TCTGGGTTAATGGCTTGCC-3'	

[1]Used with primer VA1-F.

Determination of H pylori infection

H pylori infection was determined by either serum antibodies to *H pylori*, rapid urease test (RUT) or histological examinations of biopsy specimens obtained from the antrum, cisura angularis and body of the stomach. Patients were considered to be infected with the bacterium if either serum antibodies to *H pylori* were found, the biopsy specimen was positive for RUT or the bacterium was observed in any of the hematoxylin and eosin-stained sections.

Extraction of human DNA and genotyping of cytokine polymorphisms

Human DNA was extracted from biopsy specimens using a DNA extraction kit (QIAamp DNA mini kit; Qiagen K.K., Tokyo, Japan), according to the manufacturer's instructions. Cytokine gene polymorphisms in *IL-1β* (-511 and +3954) and *IL-10* (-1082 and -592) were examined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis, as described previously[26,27] and visualized by 50 mL/L ethidium bromide staining on 30 mL/L agarose gels. The IL-1RN variable number of tandem repeat (VNTR) polymorphism was detected by PCR and visualized on 20 mL/L agarose gels with alleles being classified conventionally according to El-Omar *et al*[18] as follows: allele 1, four repeats; allele 2, two repeats; allele 3, five repeats; allele 4, three repeats and allele 5, six repeats. Because alleles 3, 4 and 5 were very rare, the alleles were classified into short (allele 2: *2) and long (alleles 1, 3, 4 and 5: L) alleles for statistical analysis, as described previously[14].

Isolation of H pylori from biopsy specimens and DNA extraction

The homogenized biopsy specimens were placed on *H pylori* selective agar plates (Helico VI agar, E-MS70, Eiken Chemical Co., Ltd., Japan) and cultured at 37°C under microaerobic conditions (100 mL/L CO_2) for five to seven days. The presence of *H pylori* colonies was confirmed by typical morphology, Gram staining and a positive urease test. From 83 patients, a total of 166 clinical isolates obtained from both antrum and corpus specimens were subjected to genomic DNA (gDNA) extraction using a DNA kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

Detection of H pylori cagA, vacA and babA2 genes by PCR

The genomic DNAs were subjected to PCR for *H pylori* genotyping analysis. Genotyping of the *cagA* gene was examined using primer pairs D008 and R008, and cagA-Fnz3 and cagA-seqR1[28,29] (Table 1). The analysis of the *vacA s* and *m* regions was carried out as previously described[13,30]. Genotyping of the *babA2* gene was examined using reported primers[31] and additional primers babA2-Fnc1 (5'-GAAAAAACATGAAAAAACACATCCTTT-CAT-3') and babA2-Rmn2 (5'-TCTGGGTTAATGGCT-TGCC-3') designed according to the following conditions: pre-heat for 2 min at 96℃, followed by 40 cycles at 96℃ for 30 s, 49℃ for 30 s, and 72℃ for 1 min. All discrepant results of *cagA* and *babA2* genotyping were confirmed by sequence analysis (Genetic Analyzer 3130 Applied Biosystems, Foster City, CA, USA) following PCR using a Big Dye Terminator v1.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analysis was performed using the Chi-square test and the Fisher's exact probability test (STATA SE (version 8) statistical software). A P-value of < 0.05 was regarded as statistically significant. Multivariate

Table 2 Characteristics of *H pylori*-positive Costa Rican patients

	Group A	Group B	*P*-value
Number of patients	101	90	
Gender (male/female)	49/52	33/57	0.81
Mean age (yr, ± SD)	50.4 ± 11.5	50.9 ± 13.6	0.99
AG-positive (%)	36 (35.6)	24 (26.7)	0.12
IM-positive (%)	17 (16.3)	6 (6.7)	0.02

analysis was performed by logistic regression (SPSS 13.0 Japanese version (SPSS Japan Inc., 2005) adjusting for gender and age. Odds ratios (OR) with 95% confidence intervals (CI) were used to study the influence of host and bacterial factors on the development of gastric cancer.

RESULTS

Comparison of gender and age of patients between groups A and B

Gender and age distribution in group A (101, 49 men, 52 women; mean ± SD, 50.42 ± 11.5 years) was not significantly different when compared with that in group B (90, 33 men, 57 women; mean ± SD, 50.87 ± 13.6 years) ($P = 0.81$ and $P = 0.99$, respectively; Table 2).

Gastric atrophy and intestinal metaplasia in groups A and B

The prevalence of gastric atrophy was higher in group A (35.6%, 36/101) than in group B (26.7%, 24/90), although the difference did not reach statistical significance ($P = 0.12$, Table 2). However, the prevalence of intestinal metaplasia was found to be significantly higher in group A (16.3%, 17/101) than in group B (6.7%, 6/90; $P = 0.02$).

Interleukin-1 and -10 polymorphisms in groups A and B

The analysis of cytokine gene polymorphisms including IL-1β-511 and +3954, IL-1RN intron 2, and IL-10-1082 and -592 did not reveal any significant difference between groups A and B (Table 3). However, when the role of cytokine polymorphisms on gastric cancer was evaluated, IL-1β+3954_T/C, IL-1RN*2/L, IL-10-592_A/A and IL-10-592_C/A were found to be individually associated with this cancer, irrespective of GCIR grouping (Table 3).

Mixed strain infection of H pylori colonized in the stomach in clinical isolates obtained from the antrum and corpus

Mixed strain infection of *H pylori* has been defined as the colonization of the same patient by *H pylori* strains harboring more than one *vacA* genotype in the same stomach[32]. The analysis of the *H pylori vacA* gene in terms of its presence/absence and genotype in each clinical isolate between the antrum and corpus in 83 patients, showed a mixed strain infection in only one patient belonging to group B and in six patients belonging to group A, of which five were diagnosed with either gastric atrophy or both gastric atrophy and intestinal metaplasia (Table 4). The *cagA* and *babA2* genes were also examined according to the same terms in those 83 patients. The prevalence of *cagA* did not differ in any of the patients while the prevalence of *babA2* differed in two patients without discordant *vacA* alleles, both belonging to group A.

H pylori cagA, vacA and babA2 genes in clinical isolates from a non-mixed infection

In the 76 clinical isolates obtained from a non-mixed infection, the prevalence of *cagA* and the prevalence of *vacA s1b* in group A (both 87.8%) were found to be significantly higher than those in group B (65.7% and 68.6%, respectively) (Table 5). A tendency for an association between *vacA m1* and GCIR variability was reported, while no significant difference was found in the prevalence of *babA2* between the groups.

Combination of cytokine polymorphisms and H pylori virulence factors in gastric cancer and non-gastric cancer patients

To investigate the influence of combined factors on the development of GC, we used the cytokine polymorphisms that were associated with GC in this study. The presence of a combination of IL-1β+3954_T/C, IL-1RN*2/L and IL-10-592_C/A slightly increased the risk of GC (adjusted OR 4.7, 1.7-13.0) when compared with patients carrying only one of the cytokine polymorphisms previously cited (Table 6). However, a combination of these polymorphisms with the addition of *H pylori vacA s1b* and *m1* genotypes, which were chosen due to their association with GC reported in a previous Costa Rican study[29], considerably increased the risk of GC (adjusted OR 7.2, 1.4-36.4). The risk was further increased when a combination of only IL-1 polymorphisms (IL-1β+3954_T/C, and IL-1RN*2/L) and *H pylori vacA s1b/m1* was evaluated (adjusted OR 9.8, 2.9-32.9).

DISCUSSION

The gastric cancer incidence rate in Costa Rica shows regional variation. Using *H pylori*-positive patients selected from high and low GCIR regions, the main objective of this study was to evaluate the potential impact of *H pylori* and/or host genetic factors on GCIR variability in Costa Rica.

The analysis of human genetic polymorphisms within the cytokine genes *IL-1β*, *IL-1RN* and *IL-10* (Table 3) as well as the ABO blood group status (data not shown) did not show any significant differences between groups A and B (high and low GCIR groups, respectively) indicating that the genetic profile of the host, including these evaluated factors, did not seem to be linked to GCIR variability in Costa Rica. It has been reported that the presence of proinflammatory cytokines induces a hypochlorhydric and atrophic response to

Table 3 Statistical analysis for several cytokine gene polymorphisms according to high gastric cancer incidence rate and gastric cancer in *H pylori*-positive Costa Rican patients

	High GCIR			Gastric cancer		
	Pos/Neg	OR (95% CI)	*P*-value	Pos/Neg	OR (95% CI)	*P*-value
Interleukin-1β-511						
T/T	28/19	1.9 (0.8-4.3)	0.136	18/47	1.6 (0.7-4.1)	0.283
T/C	50/43	1.4 (0.7-2.8)	0.317	24/93	1.2 (0.5-2.9)	0.629
C/C	23/28	1.0 reference		10/51	1.0 reference	
Interleukin-1β+3954						
T/T	2/0	-	-	-	0/2	-
T/C	56/45	1.4 (0.8-2.6)	0.237	39/101	2.1 (1.0-4.3)	0.049
C/C	43/45	1.0 reference		13/88	1.0 reference	
Interleukin-1RN intron 2						
*2/*2	20/11	2.2 (0.9-5.2)	0.078	4/31	0.7 (0.2-2.2)	0.494
*2/L	31/28	1.2 (0.6-2.3)	0.592	33/59	3.5 (1.7-7.3)	0.001
L/L	50/51	1.0 reference		15/101	1.0 reference	
Interleukin-10-1082						
A/A	52/49	1.3 (0.3-6.1)	0.766	35/101	3.2 (0.3-30.2)	0.304
G/A	46/37	1.6 (0.3-7.8)	0.551	16/83	1.8 (0.2-17.1)	0.617
G/G	3/4	1.0 reference		1/7	1.0 reference	
Interleukin-10-592						
A/A	11/12	0.7 (0.3-1.7)	0.406	10/23	3.1 (1.2-8.2)	0.022
C/A	34/31	0.9 (0.5-1.6)	0.668	26/65	3.2 (1.5-6.8)	0.002
C/C	56/47	1.0 reference		16/103	1.0 reference	

-: Unable to compute. Pos: Positive; Neg: Negative.

Table 4 Patients with discordant *H pylori vacA* and *babA2* genes from antrum and corpus biopsy specimens in the same stomach

Patient	Gene	Antrum	Corpus	Diagnosis	GCIR group
1	*vacA*	*s2/m1*	*s1b/m1*	AG	A (High GCIR)
2		*s1b/m1*	*s2/m2*	NAG	A
3		*s1b/m1*	*s2/m2*	AG	A
4		*s1b/m1*	*s2/m2*	AG + IM	A
5		*s1b/m1*	*s1b/m2*	AG + IMA	A
6		*s1b/m1*	*s2/m2*	AG	A
7		*s1b/m1*	*s1b/m2*	AG + IM	B (Low GCIR)
8	*babA2*	Pos	Neg	NAG	A
9		Neg	Pos	AG + IM	A

Table 5 Statistical analysis for the prevalence of *H pylori* genes or alleles in Costa Rican clinical isolates from groups A and B

Gene/allele	Group A (*n* = 41, %)	Group B (*n* = 35, %)	OR (95% CI)	*P*-value
cagA	36 (87.8)	23 (65.7)	3.9 (1.2-12.9)	0.026
vacA s1b	36 (87.8)	24 (68.6)	3.6 (1.1-12.1)	0.041
vacA m1	33 (80.5)	22 (62.9)	2.7 (0.9-8.0)	0.068
babA2	19 (46.3)	15 (42.9)	1.1 (0.4-2.8)	0.812

Table 6 Adjusted odd ratios with 95% confidence intervals and *P*-value for combinations of host and bacterial factors according to gastric cancer in Costa Rican *H pylori*-positive patients

Combination of factors	Gastric Cancer		
	Pos/Neg	OR (95% CI)	*P*-value
IL-1β+3954_T/C, IL-1RN*2/L, IL-10-592_C/A			
Pos	10/8	4.7 (1.7-13.0)	0.002
Neg	42/183		
IL-1β+3954_T/C, IL-1RN*2/L, IL-10-592_C/A, vacA s1b/m1			
Pos	9/2	7.2 (1.4-36.4)	0.017
Neg	40/74		
IL-1β+3954_T/C, IL-1RN*2/L, vacA s1b/m1			
Pos	18/4	9.8 (2.9-32.9)	< 0.001
Neg	31/72		
IL-1RN*2/L, IL-10-592_C/A, vacA s1b/m1			
Pos	14/9	3.0 (1.1-8.1)	0.028
Neg	35/67		
IL-1β+3954_T/C, IL-10-592_C/A, vacA s1b/m1			
Pos	21/9	4.7 (1.9-11.9)	0.001
Neg	28/67		

H pylori infection[18,20,21]. In particular, IL-1β is important in initiating and amplifying the inflammatory response to *H pylori* infection, resulting in severe inflammation possibly leading to atrophic and metaplastic changes in the gastric mucosa. An association between cytokine polymorphisms in *IL-1β* and *IL-1RN*, and gastric premalignant lesions was previously reported in a Costa Rican population[33], while carriers of IL-1β+3954_T/C and IL-1RN*2/L had an increased risk for developing gastric cancer in another Costa Rican study[34]. Likewise, our results showed that the prevalence of the proinflammatory genotypes IL-1β+3954_T/C and IL-1RN*2/L was significantly higher in gastric cancer cases than in non-cancer cases, supporting the association of polymorphisms within *IL-1β* and *IL-1RN* and gastric cancer in the Costa Rican population. Our results also showed that the carriage of IL-10-592_A/A or IL-10-592_C/A was also associated with an increased risk for gastric cancer, which has been reported previously[21]. This is the first time that polymorphisms within the cytokine gene *IL-10* have been associated with increased risk for gastric cancer in a Costa Rican population. Collectively, these

studies thus suggest that in Costa Rica, the proinflammatory cytokine genetic profile of the host is involved in the development of gastric malignancy; but, it does not seem to play a main role in GCIR variability between regions.

The evaluation of *H pylori* virulence factors revealed that all *H pylori* strains detected in gastric atrophy and/or intestinal metaplasia cases were positive for *cagA*, *vacA s1b* and *vacA m1*, supporting the association of *H pylori cagA* and *vacA* genotype distribution with gastric cancer and premalignant lesions reported in a previous Costa Rican study[29]. In addition, the prevalence of *H pylori cagA* and *vacA s1b* was significantly higher in the high GCIR group than in the low GCIR group, and a tendency for an association between *vacA m1* and GCIR variation was also detected, confirming the association between *H pylori* virulence factors, specifically *cagA*, and the GCIR variability in Costa Rican regions suggested in a previous study[24]. However, additional factors, especially not yet determined host and/or environmental and lifestyle factors could also be involved in GCIR variability in Costa Rica, as it seems unlikely that this phenomenon could be solely explained by the status of *H pylori* infection. The association between several cytokine polymorphisms and gastric cancer reported in this and past Costa Rican studies may support this possibility. Furthermore, this study also showed that carriers of IL-1β+3954_T/C, IL-1RN*2/L and IL-10-592_C/A and carriers of these polymorphisms together with the presence of *H pylori vacA s1b/m1* increased the risk of gastric cancer when compared with patients not carrying any of these factors, suggesting that a synergistic effect of a combination of bacterial and host genotypes may determine the severity of the gastritis and the final outcome of *H pylori* infection. Such a suggestion has been documented in previous studies[18,20,21].

A comparative analysis of the status of the *H pylori* genes in each clinical isolate between antrum and corpus specimens demonstrated that a mixed strain infection (discordant *vacA* genes in the same stomach) was observed in six patients from the high GCIR group, but in only one patient from the low GCIR group. Likewise, the prevalence of gastric premalignant lesions, including gastric atrophy and intestinal metaplasia, was found more frequently in the high GCIR group than in the low GCIR group. The reason for the contrasting prevalence of mixed strain infection and premalignant lesions between high and low GCIR regions is still unknown. One may speculate that during persistent infection by *H pylori* due to yet undetermined factors associated with high population density areas such as urban lifestyle stress or inadequate intake of nutrients, subjects from high GCIR regions develop more severe gastric mucosal injury with atrophic and metaplastic changes, leading to a high genetic diversity of the bacterium for adaptation to this harsh gastric microenvironment. In fact, in strains isolated from Costa Rican patients, a high frequency of recombinated *H pylori* genes (ten of ten strains) has been reported[35]. Alternatively, it does not exclude the possibility that the contrasting prevalence is caused by the difference in the frequency rate of superinfection by *H pylori* strains, which according to population density or yet undetermined factors, may occur more frequently in subjects from high GCIR regions, supposing a higher possibility of infection with the more virulent strains, which in fact have been linked with the development of gastric premalignant lesions. However, such development of premalignant changes and superinfection or genetic recombination within *H pylori* remains unclear as to which is cause and which is effect. Further investigation is essential to understand this issue, especially an investigation which includes an increased number of mixed strain infection-positive cases.

To summarize, our results demonstrated that although the carriage of proinflammatory IL-1β+3954_T/C, IL-1RN*2/L, IL-10-592_C/A and IL-10-592_A/A polymorphisms was associated with an increased risk for the development of gastric cancer, the characteristics of *H pylori* infection, in particular the status of *cagA* and *vacA* genotype distribution, seemed to play a major role in gastric cancer incidence rate variability in Costa Rican regions.

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COMMENTS

Background

Costa Rica has one of the highest age-adjusted incidence and mortality rates for gastric cancer. Costa Rica has regions with contrasting gastric cancer incidence rates (GCIR). The cause of gastric cancer is thought to be multifactorial. The risk is high in subjects infected with *Helicobacter pylori* (*H pylori*) and even higher in those infected with strains co-expressing the *cagA*, *vacA s1* and *babA2* genes. Cytokine gene polymorphisms of the host, IL-1β, IL-1RN and IL-10, in response to *H pylori* infection, have also been associated with an increased risk for developing gastric cancer.

Research frontiers

The research in this area is focused on the evaluation of host genetic factors such as interleukin (IL)-1β (-511 and +3954), IL-10 (-1082 and -592) and IL-1RN intron 2 variable number of tandem repeat (VNTR) polymorphisms in response to *H pylori* infection, and *H pylori cagA*, *vacA* and *babA2* genotype distribution on the association with the GCIR variability in Costa Rica. A total of 191 *H pylori*-positive patients were enrolled for the determination of cytokine gene polymorphisms. Clinical isolates from gastric specimens of 83 patients were used for the PCR-based genotyping of the *H pylori cagA*, *vacA* and *babA2* genes.

Innovations and breakthroughs

Cytokine polymorphisms showed no association with GCIR variability. However, gastric atrophy, intestinal metaplasia and strains with different *vacA* genotypes in the same stomach (mixed strain infection) were more frequently found in the high GC risk group than in the low GC risk group, and *cagA* and *vacA s1b* were significantly associated with high GCIR (P = 0.026 and 0.041, respectively). IL-1β+3954_T/C (OR 2.1, 1.0-4.3), IL-1RN*2/L (OR 3.5, 1.7-7.3) and IL-10-592_C/A (OR 3.2, 1.5-6.8) were individually associated with GC, and a combination of these cytokine polymorphisms with *H pylori vacA s1b* and *m1* further increased the risk (OR 7.2, 1.4-36.4).

Applications

Although a proinflammatory cytokine genetic profile showed an increased risk for developing GC, the characteristics of *H pylori* infection, in particular the status of *cagA* and *vacA* genotype distribution seem to play a major role in GCIR variability in Costa Rica.

Peer review

This study revealed that bacterial factors (i.e., *cagA* and *vacA*, but not *babA2*) are involved in regional differences in gastric cancer risk in Costa Rica, although host factors (IL-1B, IL-1RN and IL-10 polymorphisms) are associated individually with gastric cancer risk. There are interesting points found in this study in Costa Rica, where gastric risk and genetic distribution on *H pylori* are uniquely heterogeneous.

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Acute effects of *Helicobacter pylori* extracts on gastric mucosal blood flow in the mouse

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Abstract

AIM: To investigate the mechanisms underlying the reduction in gastric blood flow induced by a luminal water extract of *Helicobacter pylori* (HPE).

METHODS: The stomachs of isoflurane-anesthetized mice were exteriorized, and the mucosal surface exposed. Blood flow was measured with the laser-Doppler technique, and systemic arterial blood pressure monitored. C57BL/6 mice were exposed to water extract produced from *H pylori* strain 88-23. To investigate the role of a nerve- or iNOS-mediated pathway, we used intraluminal lidocaine and iNOS-/- mice. Blood flow response to the endogenous nitric oxide synthase inhibitor asymmetric dimethyl arginine (ADMA) was also assessed.

RESULTS: In wild-type mice, HPE decreased mucosal blood flow by approximately 30%. This reduction was abolished in iNOS-deficient mice, and by pre-treatment with lidocaine. Luminally applied ADMA resulted in reduction in blood flow similar to that observed in wild-type mice exposed to HPE.

CONCLUSION: A *H pylori* water extract reduces gastric mucosal blood flow acutely through iNOS- and nerve-mediated pathways.

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Key words: Gastric mucosal blood flow; *In vivo*; Laser-Doppler flowmetry; Mice; *Helicobacter pylori*

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INTRODUCTION

Gastric ulcer and cancer of the stomach have been shown to be associated with the bacterium *Helicobacter pylori* (*H pylori*), which colonizes up to 50% of the human population[1]. It is not known how an infection with this pathogen causes these lesions; but, disruption of the gastric protection mechanisms is certainly involved. We have previously found that a water extract of *H pylori* reduces the mucosal blood flow in rats by a mast cell- and platelet activating factor (PAF)-dependent pathway[2].

PAF is a very potent vasoconstrictor, which also mediates early leukocyte recruitment, and can cause gastrointestinal microcirculatory hypoperfusion[3,4]. PAF is released from a number of inflammatory cells, including mast cells. Mast cells function as "alarm cells" in the gastric mucosa in reaction to infectious material, evoking an inflammatory response[5]. It has been suggested that nerves in the mucosa signal to the mast cells, and inhibition with lidocaine has been found to attenuate mast cell-mediated effects[6].

Gastric mucosal blood flow has a vital role in gastric mucosal protection. A high blood flow is considered good protection against injury, as it dilutes, neutralizes, and removes hazardous substances that have penetrated the gastric mucosal barrier[7,8]. In previous studies, we have shown that high concentrations of luminal acid alone induce hyperemia without any macroscopic lesions[9,10]. Furthermore, these results suggested that epithelial inducible nitric oxide synthase (iNOS) is involved in the hyperemic response to acid, possibly signaling to afferent nerves, leading to a blood flow increase.

It is well known that *H pylori* induces iNOS expression as part of the inflammatory process[11]. Among several other functions, nitric oxide (NO) has antibacterial properties; but, despite this, *H pylori* is able to survive in the presence of the vast amount of NO that is produced. Several explanations for the survival of these bacteria have been proposed, including the production of an L-arginine analogue, asymmetric dimethyl arginine (ADMA), an endogenous inhibitor of NOS activity. In line with this, the formation of ADMA has been demonstrated in the duodenal mucosa on exposure to a water extract of *H pylori*[12].

The aim of this study was to further elucidate the acute effects of *H pylori* on the gastric mucosal blood flow and on distinct signaling pathways. We challenged normal and iNOS-deficient mice with water extracts of *H pylori*. Furthermore, lidocaine was administered intraluminally to investigate if the blood flow response to *H pylori* was nerve-mediated. In addition, we assessed the blood flow response to luminally-applied ADMA.

MATERIALS AND METHODS

All experimental procedures in this study were conducted in accordance with the guidelines of the Swedish National Board for Laboratory Animals and were approved by the Swedish Laboratory Animal Ethical Committee in Uppsala.

Mice

The animals were kept under standardized conditions of temperature (21-22℃) and illumination (12 h light/12 h darkness). They were housed in cages with mesh bottoms, and had free access to tap water and pelleted food (Lactamin, Kimstad, Sweden).

Male C57BL/6 mice (n = 29, B&K Universal, Stockholm, Sweden) were used for all experiments except for the iNOS deficient and wild-type (wt) mice (background C57BL/6 × 129SvEv). Breeding pairs of mice deficient in iNOS were kindly provided by J.S. Mudgett (Merck Research Laboratories, Rahway, NJ, USA) and JD MacMicking and C Nathan (Cornell University Medical College, New York, NY, USA). The mice were generated by gene targeting in embryonic stem cells as previously described[13]. Homozygous iNOS-deficient mutants were maintained by interbreeding the F2 generation (n = 11, Animal Department, Rudbeck Laboratory, Uppsala, Sweden). For wild-type controls male C57BL/6 × 129Sv was used (n = 6, Taconic Farms, Germantown, NY).

H pylori water extracts

The procedure for the preparation of HPE is a modification of that of Xiang *et al*[14], and has been described earlier[15]. HPE were produced from *H pylori* strain 88-23, wt (kindly provided by M. Blaser, Nashville, TN, USA). The concentrated HPE were diluted twice with a 1.8% saline solution in order to obtain a solution with isotonic properties.

Animal preparation

Anesthesia was induced by spontaneous inhalation of isoflurane (Forene®, Abbott Scandinavia AB, Kista, Sweden). The inhalation gas was administered continuously through a breathing mask (Simtec Engineering, Askim, Sweden) and contained a mixture of air, oxygen (total oxygen 40%) and about 2.4% isoflurane. Body temperature was maintained at 37-38℃ by means of a heating pad regulated by a rectal thermistor probe.

A catheter containing heparin (12.5 IU/mL) dissolved in isotonic saline was placed in the left carotid artery to monitor blood pressure. The jugular vein was cannulated for continuous infusion of a modified Ringer solution at a rate of 0.35 mL/h. In some experiments, infusion was performed intra-arterially through a Y-catheter.

The preparation of the mouse gastric mucosa for intravital microscopy has been described previously[16]. Briefly, exteriorization of the mucosa through a midline abdominal incision was followed by an incision along the greater curvature in the forestomach. The animal was placed on a Lucite table with part of the corpus of the stomach loosely draped over a truncated cone in the center of the table, with the mucosal surface facing upwards. A "mucosal chamber" with a hole in the bottom corresponding to the position of the cone was fitted over the mucosa, exposing approximately 0.13 cm^2 of the gastric mucosa through the hole. The mucosal chamber did not touch the mucosa, so as to avoid impairment of blood flow, and the edges of the hole were sealed with silicone grease. The chamber was filled with 3 mL of unbuffered 0.9% saline, maintained at 37℃ by circulation of warm water in a jacket in the bottom of the chamber. The saline was replaced at regular intervals of 10 min and the pH was measured.

Blood flow measurements

Blood flow was measured with laser-Doppler flowmetry (LDF) equipment (Periflux instruments Pf3 and Pf4001, Perimed, Stockholm, Sweden) which had previously been used to study the microcirculatory blood flow of the gastric mucosa in the rat model, as described by Holm-Rutili & Berglindh[17]. In brief, blood flow was recorded as changes in the frequency, that is, the Doppler shift, of monochromatic light from a laser probe (wavelength 633 nm; probe fiber separation 0.5 mm) illuminating a limited area of the tissue. Recorded Doppler-shifted light can be directly and linearly correlated to changes in erythrocyte flux. This flux has been shown to correlate well with gastrointestinal blood flow[17-19]. The laser probe was held in a fixed position in the chamber solution at a distance of 1-2 mm above the mucosa by a micromanipulator. With the type and position of the probe used in these studies, the laser light most likely penetrates through the entire thickness of the gastric wall[20]. However, the recorded blood flow is mainly mucosal, since the amount of back-scattered light decreases exponentially with the depth in the tissue and about 80% of the blood flow of

the stomach perfuses the mucosa. Blood flow was monitored continuously throughout the experiment.

Experimental protocol

The continuously measured blood flow was reported as percent of that during the control period, i.e. the 10 min period, prior to HPE or ADMA application, respectively. Before the experiments, the animals were allowed to stabilize for 45-55 min after surgery. The animals were divided into the following groups: Ⅰ, Control (n = 5); Ⅱ, HPE (n = 6): after a 10 min control period, HPE was applied for 2 × 20 min; Ⅲ, Lidocaine control (n = 6): lidocaine (0.5%) was applied for 3 × 20 min. The last 10 min of the first 20 min period was used as control level; Ⅳ, Lidocaine + HPE (n = 6): lidocaine (0.5%) was applied for 20 min (the last 10 min were used as control level), after which HPE mixed with lidocaine (final concentration 0.5%) was applied for 2 × 20 min; Ⅴ, iNOS control + HPE (n = 6): after a 10 min control period, HPE was applied for 2 × 20 min; Ⅵ, iNOS deficient mice + HPE (n = 6): after a 10 min control period, HPE was applied for 2 × 20 min; Ⅶ, ADMA (500 μmol/L) (n = 6): after a 10 min control period, the NOS-inhibitor ADMA was applied for 2 × 20 min; Ⅷ, iNOS deficient mice + ADMA (500 μmol/L) (n = 5): after a 10 min control period, the NOS-inhibitor ADMA was applied for 2 × 20 min.

The ADMA dose was selected from a previously published *in vivo* study[12], and the pH of the ADMA solution was adjusted with NaOH to that of the saline.

Chemicals

The modified Ringer solution was composed of 120 mmol/L NaCl (Fluka Chemie GmbH, Buchs, Switzerland), 2.5 mmol/L KCl, 25 mmol/L $NaHCO_3$, and 0.75 mmol/L $CaCl_2$ (Merck, Darmstadt, Germany). Other chemicals included heparin (Leo Pharma AB, Sweden), silicone grease (Dow Corning high vacuum grease, Dow Corning GmbH, Weisbaden, Germany), ADMA (N^G, N^G-Dimethylarginine hydrochloride, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and lidocaine (Xylocain, AstraZeneca, Södertälje, Sweden).

Statistics

All values are presented as mean ± SE. Vascular resistance was calculated as the ratio of mean arterial blood pressure (MAP, mmHg) to blood flow (perfusion units). Statistical significance was determined with ANOVA for repeated measurements, followed by Fisher' s protected least significant difference test. The level of significance was set at $P < 0.05$.

RESULTS

Groups Ⅰ and Ⅱ: Control and HPE

In control animals, the mucosal blood flow was stable during the entire measurement period (Figure 1). Water extract from *H pylori* was applied to the exposed gastric mucosa in group Ⅱ. HPE significantly decreased the blood flow to 68% ± 4%, and the resistance increased to 140% ± 12% of the pre-HPE control level (Figure 1). Mean arterial blood pressure was stable around 80 mmHg during the experiments.

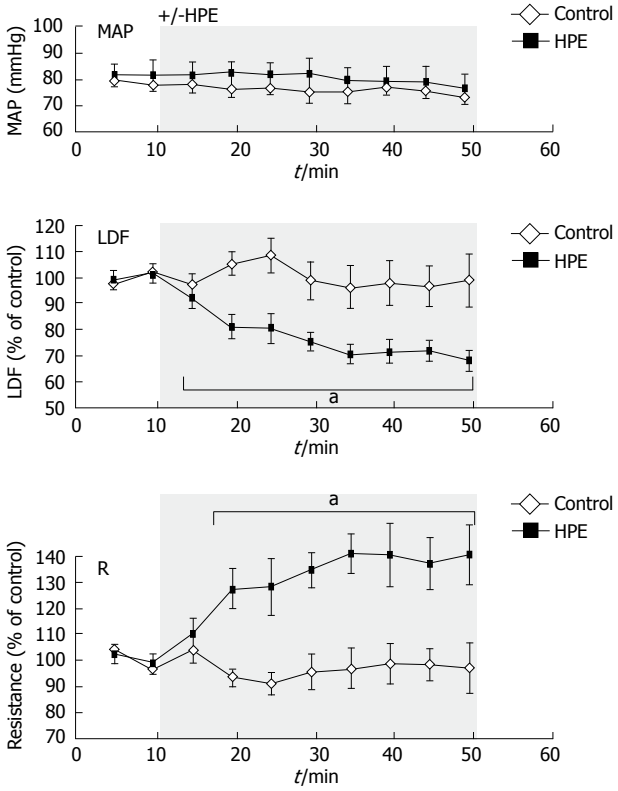

Figure 1 Blood flow measurements in control group (n = 5) and in animals exposed to HPE (n = 6). Mean arterial blood pressure (MAP) is expressed in mmHg, and blood flow (LDF) and vascular resistance (R) in percent of the control period at time-points 5-10 min. The mucosa was exposed to HPE for 40 min. Values are mean ± SE. $^aP < 0.05$ compared with control period in HPE-treated animals.

Groups Ⅲ and Ⅳ: Lidocaine experiments

Upon application of lidocaine, LDF decreased slightly, but significantly, to 83% ± 5% of the first control value (last 10-minute period of the first 20 minutes with lidocaine) (Figure 2). The blood flow also decreased slightly, but significantly, to 87% ± 5% during the application of lidocaine + HPE. Thus, the slight blood flow reduction was similar in the two groups, i.e. independently of the application of HPE, suggesting a nerve-mediated blood flow reduction. Lidocaine did not influence blood flow during the first 10 min of application, when LDF was 97% ± 3% of the mean value observed 10 min before the lidocaine application (not shown in the figure). Mean arterial blood pressure was stable during the experiments, and not significantly different between the groups.

Groups Ⅴ and Ⅵ: iNOS wt and iNOS-/-

In iNOS wt mice, the blood flow decreased significantly to 71% ± 7% upon exposure to HPE, comparable to the reduction in the control group Ⅱ. In iNOS-/- mice blood flow did not decrease during application of HPE (99% ± 6%, Figure 3), indicating that iNOS is involved in the HPE induced mucosal blood flow reduction.

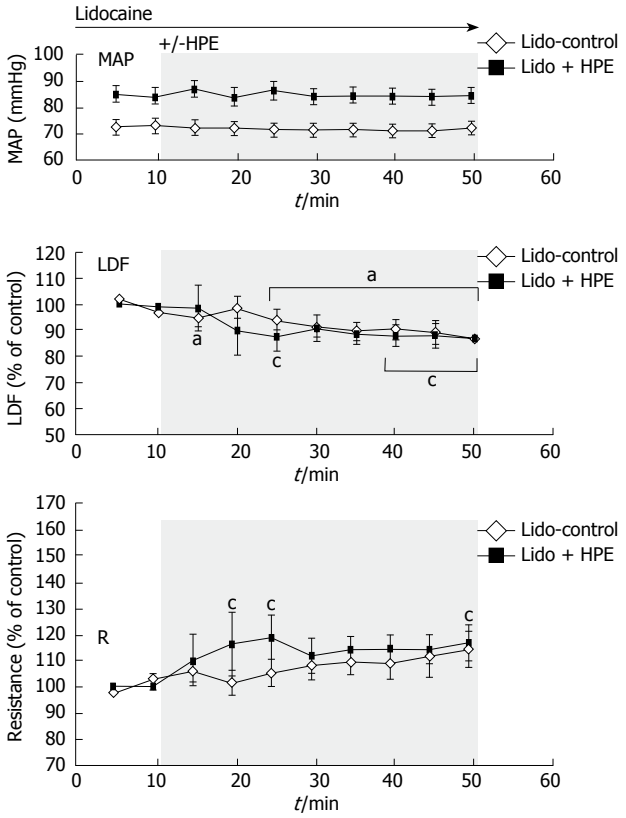

Figure 2 Blood flow measurements in the lidocaine control group (*n* = 6), and in animals treated with lidocaine and HPE (*n* = 6). Mean arterial blood pressure (MAP) is expressed in mmHg, and blood flow (LDF) and vascular resistance (R) in percent of the values of the control period at time-points 5-10 min. The mucosa was exposed to lidocaine for 10 min before the control period started, and then to lidocaine with or without HPE for 40 min. Values are mean ± SE. $^{a}P < 0.05$ compared with the control period in control animals, $^{c}P < 0.05$ compared with control period in HPE-treated animals.

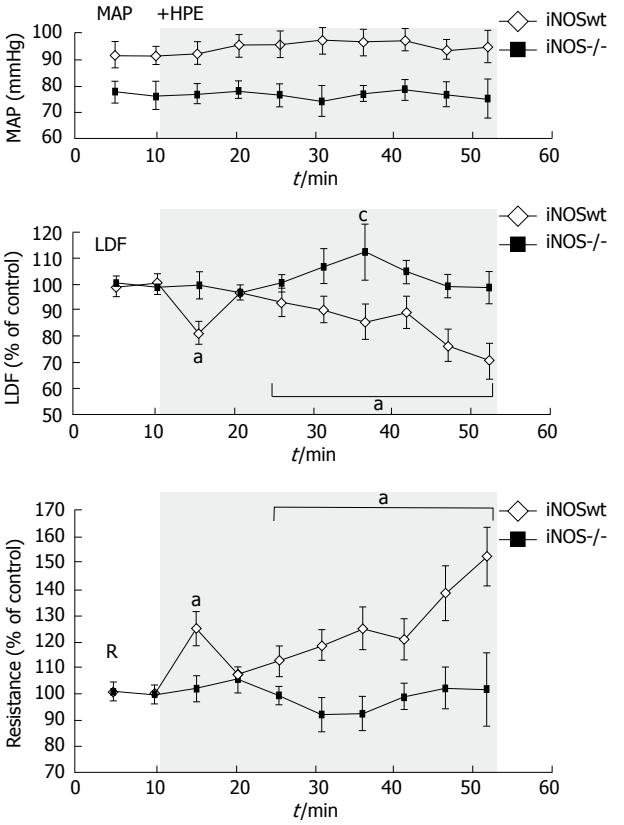

Figure 3 Blood flow measurements in iNOS wild type (*n* = 6), and iNOS-/- mice (*n* = 6), both exposed to HPE. Mean arterial blood pressure (MAP) is expressed in mm Hg, and blood flow (LDF) and vascular resistance (R) in percent of the values in the control period, at time-points 5-10 min. The mucosa was exposed to HPE for 40 min. Values are mean ± SE. $^{a}P < 0.05$ compared with the control period in control animals, $^{c}P < 0.05$ compared with control period in iNOS-/- animals.

Mean arterial blood pressure was stable during the experiments, and not significantly different between the groups.

Groups VII and VIII: ADMA experiments

During application of the NOS inhibitor ADMA in normal control mice, the blood flow decreased significantly (to 79% ± 5% of the control level, Figure 4). In iNOS-/- mice, blood flow did not decrease during application of ADMA (102% ± 7%), indicating that ADMA and HPE have the same effect in this setting. Mean arterial blood pressure was stable during the experiments and not significantly different between the groups.

MAP is usually between 70 and 90 mmHg in the C57BL/6 mice anesthetized with isoflurane. There is a tendency to higher blood pressure values in the C57BL/6x129SvEv wt mice, which was not seen in the iNOS deficient mice of the same genetic background. However, we could not find any correlation between perfusion units (LDF signal) and the blood pressure, which could explain our results. Thus, the highest LDF signal was recorded in the mice with the lowest MAP (Group III).

DISCUSSION

In this study, we have addressed the question of how the gastric pathogen *H pylori* influences the gastric mucosal blood flow and its regulation on first contact of the mucosa with water extract containing bacterial components.

When the *H pylori* water extract was applied luminally, the blood flow decreased, in conformity with our previous findings in rats. Results from the present study suggest that the HPE-induced blood flow decrease is nerve-mediated, as inhibition of local nerve activity by application of lidocaine inhibited the reduction in blood flow. In the earlier study in rats, we have also shown that the reduction in blood flow caused by HPE was inhibited both by a mast cell stabilizator, and a PAF receptor antagonist, indicating a possible effect of PAF released from degranulating mast cells[2]. A regulatory relationship between the mucosal nerves and the mast cells has been suggested, as the nerve endings are located in close proximity to the mast cells[21].

We have recently found that a constitutively expressed iNOS in the gastric surface epithelial cells is involved in the regulation of gastric mucosal blood

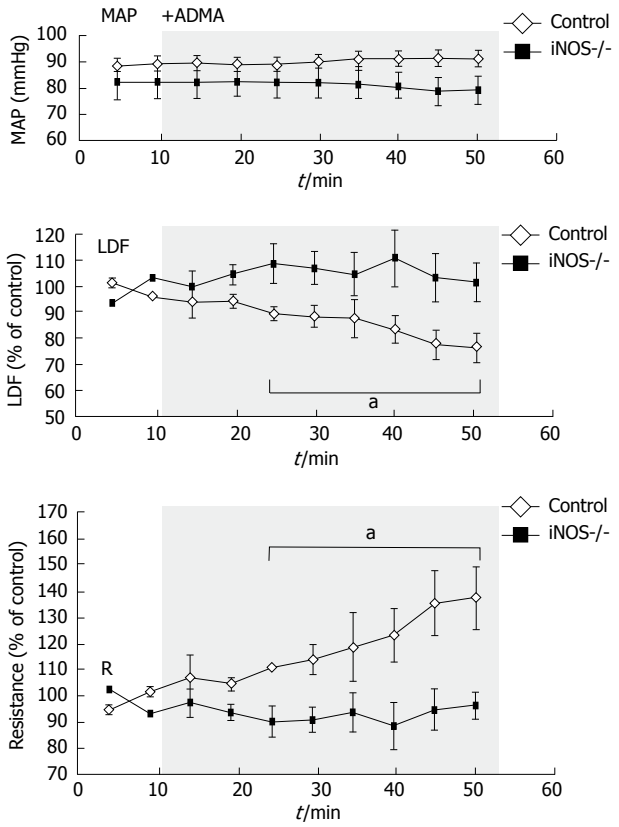

Figure 4 Blood flow measurements in the ADMA control group (*n* = 5-6), and iNOS-/- mice treated with ADMA (*n* = 5). Mean arterial blood pressure (MAP) is expressed in mm Hg, and blood flow (LDF) and vascular resistance (R) in percent of the control period at time-points 5-10 min. The mucosa was exposed to 500 μmol/L ADMA for 40 min. Values are mean ± SE. $^{a}P < 0.05$ compared with the control period.

flow[10]. The blood flow increase found upon gastric luminal application of acid was abolished in iNOS deficient mice. Holm *et al*[22] showed that iNOS played a role in the acid-stimulated HCO_3^- secretion in the duodenum. In the duodenum, HPE reduced acid-stimulated HCO_3^- secretion, and it was suggested that this effect was mediated through inhibition of the constitutively expressed iNOS by an endogenous NOS inhibitor, asymmetric dimethyl arginine (ADMA). ADMA is associated with oxidative stress[23] and the presence of HPE in the duodenum leads to increased levels of ADMA[12,24]. In the current study, the gastric mucosal blood flow reduction in response to HPE did not occur in iNOS-deficient mice. Furthermore, ADMA applied luminally caused a significant blood flow decrease in the same way as did HPE. However, when ADMA was applied luminally in iNOS-deficient mice no blood flow reduction was seen. Thus, the HPE-induced reduction in mucosal blood flow might involve inhibition of epithelial NO production. We have previously reported that the blood flow reducing effect by HPE is NO-independent. These studies were conducted on rats pretreated with the non-selective NOS inhibitor N-nitro-L-arginine (L-NNA). A reasonable explanation for the contradictory results is that L-NNA has a lower selectivity for iNOS compared with other NOS isoforms[25]. At the time of those experiments, the constitutively expressed epithelial iNOS had not been reported, and its role in blood flow regulation was consequently unheard of.

In addition to the constitutively expressed iNOS in the gastric epithelium, it has been found that *H pylori* infection induces further iNOS expression in the gastric mucosa[11], suggesting that excessive amounts of NO could be produced. Indeed, Elizalde *et al*[26] found an increase in the NO level and gastric mucosal blood flow in mice two weeks after *H pylori* infection. However, within four weeks of the infection, the NO concentration and blood flow had decreased to baseline levels[26]. Other studies have also shown lower or baseline levels of NO in infected patients[24,27]. Taken together, these results indicate that *H pylori* might alter the production of NO. The endogenous NOS inhibitor, ADMA, which is produced when HPE interacts with the mucosa, could inhibit the production of NO. Thus, even though iNOS expression is increased by *H pylori*, the epithelial NO production might be inhibited by the bacteria. An arginase produced by the bacteria has also been suggested as a strategy to reduce NO production, as it consumes the arginine-substrate for NOS[28].

In the present study, we investigated the effects of an acute exposure of products from *H pylori*. In a clinical situation it is probably of more interest to investigate the long-term effects of an infection. However, the physiological responses upon first contact with the pathogen are of great importance as it elucidates the early steps of the inflammation. We have recently investigated the effects of a chronic infection with *H pylori* in mice on different protective mechanisms in the stomach[29]. We found that the hyperemic response to luminal acid, earlier shown to be dependent on epithelial iNOS[10], was abolished in the *H pylori* infected mice. Thus, both *H pylori* infection and bacterial products have negative effects on gastric blood flow, which could be a contributing mechanism through which *H pylori* causes gastro duodenal injury.

In conclusion, we have shown that the reduction in gastric mucosal blood flow caused by a water extract of *H pylori* is mediated through iNOS- and nerve-dependent pathways. Our working hypothesis is that the epithelial iNOS is constitutively expressed and involved in the regulation of gastric blood flow in response to luminal contents. The NO produced by iNOS could, among other things, stabilize mast cells, be a signal to nerves or directly dilate blood vessels. HPE contains and/or produces ADMA, and when either of the solutions is applied onto the gastric mucosa, the NO production by iNOS is inhibited. This will remove the signal to maintain an adequate blood flow. Furthermore, reduction in NO-production will destabilize the mast cells, which can degranulate and release, for example, PAF, leading to vasoconstriction. The involvement of nerves is probably more complex, and could include both direct regulation of the blood flow and regulation

of the mast cells. Further studies are needed to elucidate which nerves are important in the blood flow effects induced by HPE.

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COMMENTS

Background

The stomach is frequently exposed to hazardous agents, and to resist this harsh environment, several protective mechanisms exist. Of special interest is the gastric pathogen *Helicobacter pylori* (*H pylori*), which causes gastritis, ulcers and cancer. However, the mechanism leading to these diseases is still unclear. It is very likely that *H pylori* negatively influences the protection mechanisms that exist in the stomach. The aim of the present study was to investigate the mechanisms underlying the reduction in gastric blood flow induced by a luminal water extract of *H pylori* (HPE).

Research frontiers

The authors studied the mechanism by which a water extract of *H pylori* reduces the gastric mucosal blood flow.

Innovations and breakthroughs

This study shows that a *H pylori* water extract reduces gastric mucosal blood flow acutely through iNOS- and nerve-mediated pathways.

Applications

The physiological responses upon the first contact with the pathogen are of great importance as they elucidate the early steps of the pathogen-associated inflammation.

Peer review

The authors showed that a *H pylori* water extract reduces gastric mucosal blood flow acutely through iNOS- and nerve-mediated pathways. The results of this research might be important for the understanding of the mechanisms of gastric inflammation.

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BRIEF ARTICLES

Adherence to surveillance guidelines for dysplasia and colorectal carcinoma in ulcerative and Crohn's colitis patients in the Netherlands

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Abstract

AIM: To study adherence to the widely accepted surveillance guidelines for patients with long-standing colitis in the Netherlands.

METHODS: A questionnaire was sent to all 244 gastroenterologists in the Netherlands.

RESULTS: The response rate was 63%. Of all gastroenterologists, 95% performed endoscopic surveillance in ulcerative colitis (UC) patients and 65% in patients with Crohn's colitis. The American Gastroenterological Association (AGA) guidelines were followed by 27%, while 27% and 46% followed their local hospital protocol or no specific protocol, respectively. The surveillance was correctly initiated in cases of pancolitis by 53%, and in cases of left-sided colitis by 44% of the gastroenterologists. Although guidelines recommend 4 biopsies every 10 cm, less than 30 biopsies per colonoscopy were taken by 73% of the responders. Only 31%, 68% and 58% of the gastroenterologists referred patients for colectomy when low-grade dysplasia, high-grade dysplasia (HGD) or Dysplasia Associated Lesion or Mass (DALM) was present, respectively.

CONCLUSION: Most Dutch gastroenterologists perform endoscopic surveillance without following international recommended guidelines. This practice potentially leads to a decreased sensitivity for dysplasia, rendering screening for colorectal cancer in this population highly ineffective.

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Key words: Colorectal cancer; Crohn's disease; Dysplasia; Guidelines; Surveillance; Ulcerative colitis

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INTRODUCTION

Patients with longstanding ulcerative colitis (UC) and Crohn's disease have an increased risk of developing colorectal cancer. This severe complication of inflammatory bowel disease (IBD) generally develops in longstanding disease. If colorectal cancer has developed in patients with IBD, the mortality rate is higher than in patients with sporadic colorectal cancer[1,2]. The lifetime prevalence of colorectal carcinoma (CRC) in UC patients is estimated to be 2% after 10 years, 8% after 20 years, and even 18% after 30 years of extensive disease[3].

Surveillance of IBD for colorectal cancer is widely practiced, and is recommended by the American Gastroenterological Association (AGA), and the British

Society of Gastroenterology (BSG) guidelines[4-6]. These guidelines aim to detect dysplasia or surgically curable cancer, and are thought to improve the prognosis. However, the reduction in mortality in patients with IBD and colorectal cancer through surveillance has still to be proven in large prospective randomized controlled trials. Table 1 gives an overview of the key elements in screening patients with long-standing, extensive IBD as recommended by the AGA[6]. These recommendations are applicable to both UC and Crohn's colitis. Since dysplastic lesions in these patients often present as flat or depressed abnormalities, surveillance colonoscopies should be performed in combination with an extensive biopsy protocol. High-grade dysplasia (HGD) in flat mucosa or a Dysplasia Associated Lesion or Mass (DALM) is considered an indication for colectomy when the pathological findings are confirmed by a second experienced pathologist. There is still no consensus on management in cases of unifocal or multifocal low grade dysplasia (LGD) in flat mucosa. What complicates the issue, as earlier studies have indicated, is that there seems to be difficulty in confirming dysplasia by the pathologist[7,8]. The management of the different forms of dysplasia varies from no management or intensifying the screening program to immediate colectomy. When advising a patient on colectomy, other factors like age, a coexisting diagnosis of primary sclerosing cholangitis (PSC) or a family history of colorectal cancer should be taken into account.

As there are no current Dutch guidelines available regarding surveillance of IBD, we presumed that Dutch Gastroenterologists (GEs) would adopt the current AGA guidelines or other relevant guidelines. To investigate the effect of surveillance guidelines on the detection of dysplasia or colorectal cancer, the first step is to study adherence to these guidelines in clinical practice. This study was designed to assess whether screening programs and recommendations set by e.g. the AGA are used by Dutch GEs for patients with ulcerative or Crohn's colitis and whether the guidelines are followed correctly.

Table 1 Key elements in screening patients with long-standing, extensive colitis, adapted from the AGA and BSG guidelines

	Key element
Surveillance colonoscopy	Colonoscopy with systematic biopsies Perform surveillance every 1 to 2 yr After 8 to 10 yr of disease in those with pancolitis After 15 yr of disease in those with left-sided colitis
Biopsy protocol	Biopsies every 10 cm in all 4 quadrants. Additional biopsies of strictures and mass lesions other than pseudopolyps Polyps that appear potentially dysplastic remove by polypectomy with biopsy of adjacent flat mucosa
Dysplasia	If HGD or multifocal low-grade dysplasia is found in flat mucosa refer for colectomy Presence of low-grade dysplasia, particularly if it is unifocal: no consensus DALM is an indication for colectomy
Other factors of consideration to advise on colectomy	Ongoing colitis-related symptoms Life expectancy Duration, severity and extent of colitis A personal history of primary sclerosing cholangitis A family history of colorectal cancer Discussion around the time of surveillance of benefit, harms, and short comings of colonoscopy surveillance

MATERIALS AND METHODS

After reviewing the widely used guidelines of the AGA, the American College of Gastroenterology and the American Society for Gastrointestinal Endoscopy, in addition to the relevant literature, a questionnaire was developed. The questionnaire was focussed on the use, feasibility and ability to follow the screening guidelines, and contained 18 multiple choice questions and one open question. We asked the invited GEs if they practised surveillance in IBD patients, if they used one of the recommended guidelines and, if not, the reason why not. The other questions, all of a multiple choice design, were divided in four subgroups: (1) start of surveillance; (2) time interval between surveillance endoscopies; (3) biopsy protocol; and (4) management of dysplasia.

In the Netherlands, surveillance endoscopies are usually performed by gastroenterologists. As almost all gastroenterologists are also registered as members of the Dutch Gastroenterology Association, we only included registered gastroenterologists, with the exception of gastroenterologists that were still in training or did not work in Dutch hospitals (n = 34). To ensure the reliability of the answers provided, the questionnaire was anonymous, and to guarantee privacy of the hospitals involved, no questions were asked on the type of hospital (e.g. teaching, non-teaching). The questionnaire could be completed in less than 5 min. To increase the response rate, a reminder was sent to all GEs after 3 wk. Results were tabulated after the second letter. Data were statistically analysed using the Statistical Package for the Social Sciences or SPSS (version 12.0.2) using frequencies.

RESULTS

Of the 244 questionnaires, 153 were returned, yielding an overall response rate of 63%. Five GEs were excluded from further analysis: 2 were recently retired, 1 was currently working in another country and 2 stated they had no experience with IBD patients. The remaining 148 were analysed (61%).

Reasons for non surveillance

Seven (5%) GEs did not provide surveillance for their IBD patients. Four GEs indicated that they would only include IBD patients in a surveillance program in cases with a positive family history of CRC, while 2 considered the available evidence in the literature to be insufficient

to justify screening in this patient category. One GE did not explain his or her motivation.

Surveillance of patients

Of all responding GEs, 95% (n = 141) provided surveillance for their IBD patients. Of these GEs, 46% stated that they did not follow any of the recommended guidelines, 27% followed the AGA guidelines, and 27% used a local protocol. Only 2 GEs followed the British guidelines. All GEs performed surveillance in UC patients, and 65% performed surveillance in patients with Crohn's colitis.

All further results are based on the 95% (n = 141) of GEs who performed surveillance on IBD patients.

Start surveillance

The start of surveillance depends on which time point is taken as the starting point, i.e. the diagnosis of IBD. This can be crucial as there can be a substantial delay between the onset of symptoms and diagnosis of IBD. Sixty nine percent started surveillance from the moment a firm diagnosis of Crohn's disease or UC was made, whilst 31% started surveillance from the onset of IBD symptoms.

When asked how the duration of disease influenced their policy on commencement of surveillance for pancolitis or left-sided colitis, 53% of the GEs stated that they initiated colonoscopic screening for pancolitis after 8 to 10 years while 44% started screening after 10 to 15 years for left-sided colitis, which is in line with the AGA guidelines (Figure 1A).

The extent of colitis also plays a role[3,9]. Six percent of the GEs would screen patients with disease activity limited to the rectum, 68% would screen patients with left-sided colitis, and 26% would screen only in the case of pancolitis.

When asked what other factors would influence their screening protocol, 42% of the GEs mentioned PSC, 30% mentioned a positive family history of CRC, co-morbidity and general health, while 28% of the GEs did not take any factor into consideration.

Time interval between surveillance

Fifty-three percent of the GEs performed colonoscopic surveillance every 1 to 2 years, which is consistent with the AGA guidelines, whilst 22% performed surveillance once every 3 years in the second decade, once every 2 years in the third decade, and once a year in the fourth decade which is consistent with the British guidelines, 14% performed surveillance once every 3 years, and the rest of the GEs performed surveillance at different intervals without a specific protocol.

Biopsy protocol

Forty three percent of the respondents stated that they take biopsies every 10 cm in every quadrant. The remaining GEs took 2 to 4 biopsies from different bowel segments (cecum, colon ascendens, colon tranversum, colon descendens, sigmoid, and rectum). Only 27% of all the respondents obtained more than 30 biopsies per colonoscopy, as recommended by the guidelines and Rubin *et al*[10]. Overall, the mean number of biopsies taken was 24 (Figure 1B).

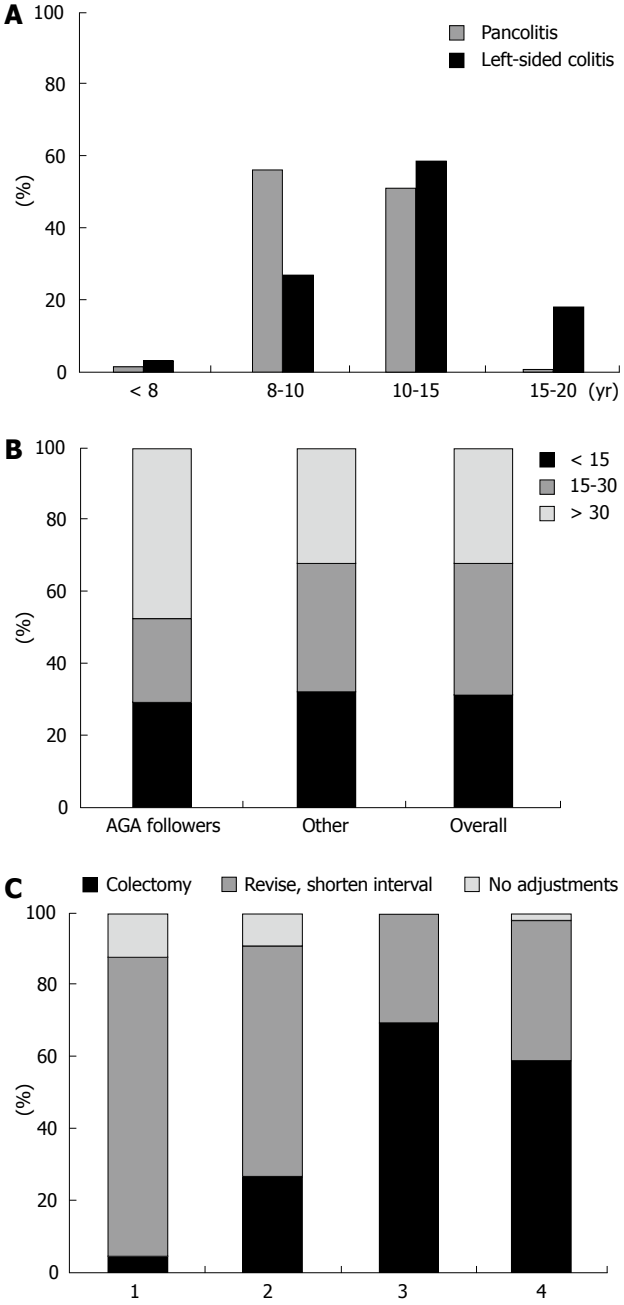

Figure 1 Different aspects of adherence to surveillance guidelines by Dutch GEs. A: Starting point of surveillance for colitis patients. Bars represent the percentage of Dutch Gastroenterologists who would start surveillance in patients with pancolitis (grey) or left-sided colitis (black) after different intervals following diagnosis (in years); B: Number of biopsies taken per colonoscopy. The first column represents clinicians claiming to follow the AGA guidelines in their clinic ("AGA followers"), the second column represents clinicians following other house protocols ("Other"). In the third column the overall results are depicted. Number of biopsies are shown per category (less than 15 biopsies in black, 15 to 30 biopsies in grey and 30 or more biopsies in pale grey); C: Management of Dysplasia. Columns represent unifocal LGD (1), multifocal LGD (2), HGD (3) and DALM (4). The percentages of doctors who would perform a colectomy (black), a revision of the pathology and/or shortening of the surveillance protocol (grey) or would not make any adjustment (pale grey) are shown.

Management of dysplasia

When asked which policy was adopted during the follow-up period when unifocal LGD, multifocal LGD, HGD, DALM or CRC was observed, the GEs responded as shown in Figure 1C. If dysplasia was confirmed, 47% of the GEs advised that they would revise the histopathology, while 40% advised that they would obtain new biopsies, and 13% advised that they would do both. In the case of a DALM, 60% of the GEs would take biopsies from the lesion and the surrounding area, 36% would take biopsies from the lesion only, and 4% would remove the lesion endoscopically.

If a subtotal colectomy was performed, 83% would screen the rectum, and 22% of the 83% would screen once every 4 to 5 years, 54% would screen once every 2 years, while 7% would screen, but not on a regular basis.

DISCUSSION

Most Dutch gastroenterologists perform endoscopic surveillance without following international recommended guidelines. Although, surveillance guidelines are widely used, there is no agreement on surveillance guidelines or a national surveillance protocol in Netherlands. We studied the surveillance practice of Dutch GEs using a postal questionnaire. Overall, 153 out of 244 questionnaires were returned, a response rate which was comparable to most questionnaire-based studies directed at physicians[11]. We, therefore, assume that this study gives a representative overview of the use, feasibility, and ability to follow surveillance guidelines in Netherlands and that the results reflect the practice of a representative number of Dutch GEs. It is possible that the GEs who answered this questionnaire were more in favour of surveillance than GEs that did not fill out the questionnaire, which might have led to information bias. All GEs who provided surveillance agreed that screening patients with UC is necessary. It appears from the literature that not only UC, but also Crohn's colitis is associated with an increased risk of colorectal cancer and, therefore, most experts recommend the use of the same guidelines for both UC and Crohn's colitis[4,6]. However, only 65% of Dutch GEs provide surveillance for patients with Crohn's colitis.

We compared our results with the guidelines set by the AGA. Firstly, we observed a large discrepancy between answers from GEs regarding the principle of surveillance in general, and the responses they provided related to their exact employment of surveillance in daily practice. Furthermore, although both pancolitis and left-sided colitis are associated with an increased risk of CRC[3], a quarter of the GEs do not provide surveillance for patients with left-sided colitis. On the other hand, a small group of GEs considered disease activity limited to the rectum an indication for screening, although there are no data to support the concept that proctitis increases the risk of CRC. All these inconsistencies could result in inefficient surveillance and missed dysplasia or even cancer.

The time between onset of symptoms and confirmed diagnosis of IBD can also differ substantially. Although there is no consensus on this subject, this difference in opinion might potentially lead to a delay in screening of months or even years.

Another important aspect of surveillance for CRC in IBD is adherence to the biopsy protocol. The median number of biopsies taken amongst Dutch GEs was 24 (range 10-40), while only 27% of the GEs approached the recommended number of 33 random biopsies. This number of biopsies was estimated to be necessary to detect possible dysplasia with a sensitivity of 90%[10]. A similar questionnaire-based study in New Zealand showed a median number of 17 biopsies[12]. This again, will inevitably lead to a pronounced decrease in sensitivity, rendering the surveillance tool ineffective.

If dysplasia is detected histopathologically, there seems to be uncertainty as to how to proceed with clinical decision-making. In the case of unifocal LGD, most of the Dutch GEs would have the histopathology revised, and would shorten the time interval to the next colonoscopy. If multifocal LGD is detected, Dutch GEs hesitate to recommend immediate colectomy, but prefer to revise the histopathology by consulting another pathologist or order a new colonoscopy with biopsies. Another suggested option was to shorten the time interval between screenings. Although controversy exists regarding the treatment policy which should be adopted after diagnosing dysplasia in patients with colitis, most experts agree that in all cases of confirmed dysplasia a colectomy should be recommended. Even the presence of LGD, which is associated with CRC in 21.4%-54%, can be considered an indication for surgery[4,6]. There is a disconcertingly low referral rate for colectomy amongst Dutch GEs, and even more so when findings are compared with 3 similar questionnaire-based studies in New Zealand, United Kingdom and Canada[12-14]. It is remarkable that the referral rate is higher for LGD and much lower for HGD and DALM compared with the other studies. The difficulty in confirming dysplasia, the lack of consensus for management of LGD, and the underestimation of the potential risk of LGD and HGD may contribute to the cautious management of LGD and HDG in Dutch GEs. Another reason could be that in the UK and the USA, the guidelines have already been implemented, which would explain the higher referral rate of cases with HGD in these countries.

In conclusion, 95% of Dutch GEs offer some form of surveillance, but most do not adhere to international guidelines. This leads to a decreased sensitivity for dysplasia, rendering this surveillance practice less effective. Furthermore, the management of dysplasia, even in cases of DALM, is inconsistent and will potentially lead to delays in the diagnosis of carcinomas. We suspect that this deviation from the guidelines is a general phenomenon in clinical practice, and is not only restricted to the Netherlands. Implementation of national guidelines and education of GEs concerning all aspects of colonoscopic surveillance is of great importance and will lead to a more consistent and efficient surveillance practice.

COMMENTS

Background

Patients with longstanding ulcerative colitis (UC) and Crohn's colitis harbour an increased risk of developing colorectal cancer. It is generally agreed that screening and surveillance is a rational strategy in these patients, although the optimal screening strategy and approach to managing outcomes is still being debated.

Research frontiers

Data from recent studies and new endoscopic techniques have changed the concepts on which surveillance guidelines have been built. Still, surveillance will depend on colonoscopy and requires commitment from both patients and gastroenterologists (GEs). Implementation of widely accepted guidelines is indispensable in realising optimal efficacy of a surveillance protocol. The challenge is to acquire nationwide support; only this will lead to a more consistent and efficient surveillance practice.

Innovations and breakthroughs

The authors report that most Dutch GEs offer some form of surveillance, although the majority do not follow international guidelines. This potentially results in the delayed diagnosis of advanced neoplasia in these patients, such as Dysplasia Associated Lesion or Mass (DALM), high-grade dysplasia (HGD) and colorectal carcinoma (CRC). The data are in line with studies from the UK, Canada and New Zealand, and call for more awareness on the level of national gastroenterology associations and GEs alike.

Applications

Implementation of national guidelines and education of GEs concerning all aspects of colonoscopic surveillance is of great importance and will probably lead to a more consistent and efficient surveillance practice.

Peer review

In this manuscript, authors describe the results of their questionnaire regarding the association between the risk of CRC in patients with inflammatory bowel disease (IBD) and endoscopic screening. They conclude that implementation of national guidelines and education of GEs concerning all aspects of colonoscopic surveillance in IBD patients is of great importance. The findings are of interest.

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BRIEF ARTICLES

Features of hepatocellular carcinoma in cases with autoimmune hepatitis and primary biliary cirrhosis

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Abstract

AIM: To characterize the clinical features of hepatocellular carcinoma (HCC) associated with autoimmune liver disease, we critically evaluated the literature on HCC associated with autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC).

METHODS: A systematic review of the literature was conducted using the Japana Centra Revuo Medicina database which produced 38 cases of HCC with AIH (AIH-series) and 50 cases of HCC with PBC (PBC-series). We compared the clinical features of these two sets of patients with the general Japanese HCC population.

RESULTS: On average, HCC was more common in men than in women with AIH or PBC. While many patients underwent chemolipiodolization (CL) or transcatheter arterial embolization (TAE) (AIH-series: P = 0.048 (*vs* operation), P = 0.018 (*vs* RFA, PEIT); PBC-series: P = 0.027 (*vs* RFA, PEIT), others refused therapeutic interventions [AIH-series: P = 0.038 (*vs* RFA, PEIT); PBC-series: P = 0.003 (*vs* RFA, PEIT)]. Liver failure was the primary cause of death among patients in this study, followed by tumor rupture. The survival interval between diagnosis and death was fairly short, averaging 14 ± 12 mo in AIH patients and 8.4 ± 14 mo in PBC patients.

CONCLUSION: We demonstrated common clinical features among Japanese cases of HCC arising from AIH and PBC.

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Key words: Autoimmune hepatitis; Autoimmune liver disease; Hepatocellular carcinoma; Literature review; Primary biliary cirrhosis

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INTRODUCTION

Autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerotic cholangitis (PSC) form the triad of autoimmune liver diseases. As defined by Mackay *et al*[1], AIH is a chronic active hepatitis resulting from several distinct autoimmune phenomena. While the anti-inflammatory effects of steroid therapy for this disease may inhibit the promotion of liver carcinogenesis, hepatocellular carcinoma (HCC) does occur rarely in patients with this condition (in about 0.5% of AIH cases)[2,3].

In contrast to AIH, PBC results from an autoimmune mechanism causing chronic cholestasis and chronic non-suppurative destructive cholangitis in medium sized intrahepatic bile ducts[4]. Rare cases of HCC arising from PBC have been reported to date. However,

this association is rare (affecting between 0.3% and 4.22% of cases)[5-11], because a PBC patient's ability to produce regenerative nodules is weak[5-9,12]. Additionally, PBC is pathologically characterized in chronic non-suppurative destructive cholangitis (CNSDC), and the main inflammatory lesions associated with PBC are not hepatocytes, but cholangiocytes, which may be one of the reasons why the incidence of HCC with PBC is low, especially at the early stage when cirrhotic and fibrotic changes do not progress. Recently, reports have suggested that the prevalence of HCC arising from both AIH and PBC is higher than previously believed. In 2001, Caballeria *et al*[13] found that the incidence of HCC in patients with advanced PBC (Scheuer histological stage Ⅲ or Ⅳ) was 11.1%, approximating the 15% incidence in patients with HCV-related cirrhosis (RR 0.812, 95% CI 0.229-2.883). The clinical features of HCC associated with AIH and PBC, however, have not yet been extensively described. Here, we performed a systematic literature review of HCC cases associated with AIH and PBC in Japan, a country with a high burden of autoimmune liver disease. We conducted a critical analysis of case reports to find common themes in the demographic and clinical histories of patients with HCC associated with AIH and PBC.

MATERIALS AND METHODS

We performed a systematic literature review of case reports published in Japan and listed in the Japana Centra Revuo Medicina database, version 3 (systematic literature search system through a computer web site for Japanese literature), using the keywords "hepatocellular carcinoma", "autoimmune hepatitis", and "primary biliary cirrhosis". The database search was limited to the period between 1990 (when the hepatitis C virus was first detected) to the present. The quality of this database available for analysis is thoroughly well-documented. In total, 38 cases of HCC associated with AIH, and 50 cases of HCC associated with PBC were identified. No cases were duplicated, and patients were identified across multiple Japanese medical centers. Most patients in the series had been diagnosed with autoimmune liver disease before HCC was identified. Several cases also presented with co-factors of liver damage and HCC development other than AIH or PBC, such as excessive alcohol intake, HBV, or HCV infection. However, no cases had evidence of hemochromatosis or α1-antitrypsin deficiency. The demographics of these two groups were recorded based on gender, age, period of medical observation, and history of blood transfusion or excessive alcohol intake. Clinical data was also recorded to determine noncancerous pathologies of the liver, HBV or HCV infection status, serum α-fetoprotein (AFP) level, maximal tumor size, history of HCC therapy, clinical outcomes, and cause of death. Cases that did not include a description of alcohol intake were assumed not to have histories of excessive alcohol intake.

We confirmed that all 38 identified cases of HCC associated with AIH met generally accepted international criteria for diagnosis of AIH[14]. Scoring was performed prior to AIH therapy initiation; all scores were greater than 10, and thereby classified as either "probable AIH" or "definite AIH".

Because no internationally accepted diagnostic criteria yet exists for PBC, we utilized the Japanese standard criteria for PBC diagnosis, a standard first proposed in 1992 by a clinical study group supported by the Japanese Ministry of Welfare. According to this standard, PBC diagnosis requires that cases meet at least one of the following criteria: (1) pathologic evidence of CNSDC and positive anti-mitochondrial antibody (AMA) or anti-PDH antibody titers, (2) positive AMA or anti-PDH antibody titers and non-CNSDC pathology compatible with PBC, or (3) no liver biopsy, but, positive AMA or anti-PDH antibody titers and a clinical picture and clinical course compatible with PBC. We confirmed that all 50 identified cases of HCC associated with PBC met the above diagnostic criteria. Six of 50 (12.0%) HCC cases with PBC met the third criteria for PBC, and 44 of 50 (88.0%) cases met the first or second criteria for PBC. The third criteria for PBC remain ambiguous, and it is really hoped that internationally accepted criteria will be determined for PBC diagnosis.

If a case met both generally accepted international criteria for diagnosis of AIH, and the Japanese standard criteria for PBC diagnosis, we diagnosed the case as overlap syndrome. We had two cases of overlap syndrome, and excluded these cases from our analysis.

We did not include a control group, but used the general HCC population in Japan for comparison[15].

Statistical analysis

Intention-to-treat analyses were used throughout, and statistical analysis for categorical comparisons of the data was performed using the program ystat2006.xls for Windows/Macintosh (Igaku Tosho Shuppan Corporation, Tokyo, Japan). We used the χ^2 test and Fisher's exact test for categorical comparisons between patients with HCC associated with AIH or PBC and HCC patients without associated autoimmune disease[15]. The following variables were assessed: gender, HBV or HCV co-infection, history of blood transfusions, history of excessive alcohol intake, positivity for serum-AFP and clinical outcomes. Because the baseline male to female ratio of AIH and PBC was 1:7 and 1:9, respectively, we performed the χ^2 test for males and females separately. We also used the χ^2 test with or without the Yates correction for categorical comparisons of pathological findings of noncancerous lesions of the liver, HCC therapy choices, and cause of death. Where significant differences were noted, χ^2 tests or Fisher's exact tests were repeated with all categorical combinations, using Bonferroni corrections for multiple comparisons. Two tailed Mann-Whitney *U*-tests and *F*-tests were performed at the 5% significance level only for comparisons between HCC patients with AIH and PBC, as the following variables were unavailable for the general HCC

Table 1 Developement period of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/ General-HCC patients	PBC-series/ General-HCC patients	AIH-series/ PBC-series
Observation period (mean ± SD)	Total: 38 1 yr 1 mo-23 yr (10 yr 6 mo ± 6 yr 7 mo)	Total: 49 3 mo-24 yr (9 yr 4 mo ± 6 yr 4 mo)	NA	NA	NA	$P = 0.307$ ($P = 0.815$)
Interval between liver damage and HCC diagnosis (mean ± SD)	Total: 34 0-22 yr 9 mo (10 yr 2 mo ± 6y 5 mo)	Total: 40 0-24 yr (9 yr 9 mo ± 7 yr 0 mo)	NA	NA	NA	$P = 0.740$ ($P = 0.688$)
Period from HCC development to death (mean ± SD)	Total: 18 2 mo-3 yr (1 yr 2 mo ± 12 mo)	Total: 16 0-5 yr (8.4 ± 14 mo)	NA	NA	NA	$P = 0.047$[a] ($P = 0.401$)

The *P*-value above was calculated from the Mann-Whitney *U*-test and the *P*-value below, indicated in parentheses, was calculated from the *F*-test. [a]$P < 0.05$, Statistically significant. HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; SD: Standard deviation; NA: Not available.

Table 2 Analysis on gender and age of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers (%)			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/ General-HCC patients	PBC-series/ General-HCC patients	AIH-series/ PBC-series
Gender						
Actual number	Total: 38	Total: 50	Total: 16743			
Male	7 (18.4)	13 (26.0)	12025 (71.8)			
Female	31 (81.6)	37 (74.0)	4718 (28.2)	$P = 0.149$[1]	$P = 0.512$[1]	$P = 0.244$
Relative number	Total: 38	Total: 50				
Male	23.3 (61.3)	38.0 (76.0)				
Female	14.7 (38.7)	12.0 (24.0%)				
Age at HCC diagnosis	Total: 38	Total: 50	Total: 16743			
(mean ± SD)	(67.61 ± 8.58)	(68.54 ± 9.30)	NA			
< 40 s	0 (0)	2 (4.0)	761 (4.6)	NA	NA	$P = 0.410$
50 s	8 (21.0)	6 (12.0)	2818 (16.8)			($P = 0.614$)
60 s	16 (42.1)	21 (42.0)	6179 (36.9)			
70 s	9 (23.7)	14 (28.0)	5976 (35.7)			
< 80 s	5 (13.2)	7 (14.0)	1009 (6.0)			

The *P*-value above was calculated from the Mann-Whitney *U*-test and the *P*-value below, indicated in parentheses, was calculated from the *F*-test. [1]The *P*-value was calculated from the relative numbers. HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; SD: Standard deviation; NA: Not available.

population: interval between liver damage and HCC diagnosis, interval from HCC diagnosis to death, age at HCC diagnosis, serum-AFP levels, maximum tumor size and number of HCC loci. Because the patient sample size in each group was greater than 20, we chose to use *P*-values calculated from the asymptotic distribution. The total number of cases in each patient group did not include cases for which categorical data were unknown (Table 1).

The statistical analysis for survival among HCC patients with AIH and PBC was performed on a personal computer with the statistical package SPSS for Windows (version II, SPSS Inc., Chicago, IL, USA). Because there were too few published cases of HCC arising from AIH or PBC, however, differences in survival between patient groups could not be calculated.

RESULTS

The intervals between HCC diagnosis and death for HCC patients with AIH (14 ± 12 mo) and PBC (8.4 ± 14 mo) was notably shorter than among general HCC patients in Japan (77.5% 1-year survival, 52.5% 3-year survival, and 35.4% 5-year survival)[15]. As shown in Table 1, the survival interval for HCC patients with PBC was also significantly shorter than that for patients with AIH ($P = 0.047$).

Among HCC cases associated with AIH, the actual male to female ratio was 7:31. Because AIH patients in Japan are predominantly female (7:1), the corrected risk ratio for HCC among male AIH patients was 1.6:1 relative to females, and the male to female ratio of the relative numbers was 23.3:14.7 (Table 2). The majority of Japanese PBC patients are also female, outnumbering

Table 3 Clinical status of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers (%)			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/General-HCC patients	PBC-series/General-HCC patients	AIH-series/PBC-series
History of blood transfusion	Total: 29	Total: 38	Total: 12602	$P = 0.040$[a]	$P = 0.581$	$P = 0.041$[a]
+	3 (10.3)	13 (34.2)	3633 (28.8)			
-	26 (89.7)	25 (65.8)	8969 (71.2)			
History of excessive alcohol intake	Total: 38	Total: 50	Total: 14694	$P = 0.812$	$P = 0.056$	$P = 0.352$
+	1 (2.6)	5 (10.0)	3271 (22.3)			
-	37 (97.4)	45 (90.0)	11423 (77.7)			
Co-infection	Total: 33	Total: 40	Total: 4121			
HBV (prior) +	2 (6.1)	10 (25.0)	2138 (51.9)	$P < 0.001$	$P < 0.001$	$P = 0.025$
HBV (prior) –	31 (93.9)	30 (75.0)	1983 (48.1)			
Co-infection	Total: 38	Total: 49	Total:16492			
HCV +	3 (7.9)	10 (20.4)	11488 (69.7)	$P < 0.001$	$P < 0.001$	$P = 0.044$
HCV -	35 (92.1)	39 (79.6)	5004 (30.3)			
Pathological findings of noncancerous lesion of the liver	Total: 31	Total: 44	Total: 4941	$P = 0.163$	$P = 0.007$[b]	$P = 0.489$
NL, CH, LF	13 (41.9)	15 (34.1)	2691 (54.5)			
LC	18 (58.1)	29 (65.9)	2250 (45.5)			

HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NL: Normal liver; CH: Chronic hepatitis; LF: Liver fibrosis; LC: Liver cirrhosis.

males by 9:1. The relative risk ratio for HCC among males with PBC was 3.2:1 relative to females, and the male to female ratio of the relative numbers was 38:12 (Table 2). No significant differences in male to female ratios were noted between the three patient groups ($P = 0.149$, $P = 0.512$, $P = 0.244$, respectively).

Among the HCC cases associated with AIH, only three (10.3%) had a history of blood transfusions, while 13 (34.2%) of the cases with PBC had such a history. Among all Japanese patients with HCC, 3633 (28.8%) had a history of blood transfusions[15]. The proportion of HCC cases associated with AIH having a history of blood transfusions was significantly lower than that of the general HCC cases in Japan ($P = 0.040$), and the proportion of HCC cases associated with PBC having a history of blood transfusions was significantly greater than that of the HCC cases associated with AIH ($P = 0.041$, Table 3).

Similarly, only one case (3.1%) of HCC associated with AIH had a history of excessive alcohol intake, while five (20.0%) cases associated with PBC had such a history ($P = 0.352$, Table 3). Among all Japanese patients with HCC, 3271 (22.3%) had a history of excessive alcohol intake[15].

While prior infection with HBV was relatively rare among AIH patients (6.1%), it was much more prevalent among patients with PBC (25.0%, $P = 0.025$). Similarly, 7.9% of AIH patients tested positive for HCV, as compared to 20.4% of PBC patients ($P = 0.044$). The population of Japanese HCC patients without autoimmune liver disease had significantly higher rates of both HBV and HCV co-infection ($P < 0.001$, Table 3).

Among the HCC cases associated with AIH, 18/31 (58.1%) were found to have cirrhosis on examination of liver biopsy samples or resected samples at operation. In contrast, 29/44 (65.9%) of the HCC cases associated with PBC were found to have cirrhotic liver tissue. Within the general HCC population in Japan, 2250 of the 4941 cases for which liver specimens were available (45.5%) showed evidence of cirrhosis[15]. While the proportion of liver cirrhosis among HCC cases associated with PBC was significantly greater than that in the general HCC population in Japan ($P = 0.007$), no statistical significance in the prevalence of cirrhosis was found between AIH-associated HCC and general HCC patients ($P = 0.163$, Table 3).

The numbers and positive ratios of the AIH-series, PBC-series and general-HCC patients were 22/37 (59.5%), 34/47 (72.4%) and 10075/15831 (63.6%), respectively. No significant differences in positive ratios of serum-AFP were noted between the three patient groups ($P = 0.597$, $P = 0.216$, $P = 0.214$, respectively, Table 4). AFP levels at diagnosis were 2340.2 ng/mL (range 1-49100 ng/mL) among patients with AIH, and 854.2 ng/mL (range 4.2-14646 ng/mL) among patients with PBC. The maximum size of the primary hepatic tumor at diagnosis was 3.97 cm (range 1.0-10.0 cm) among patients with AIH and 3.51 cm (range 1.0-8.8 cm) among PBC patients (Table 4). Due to lack of available data, we could not compare serum AFP levels, tumor sizes and numbers of HCC loci between the autoimmune-associated HCC cases and the general HCC cases in Japan. However, we found that serum AFP level did not vary widely, and that maximum tumor size and number of HCC loci were considerably lower in patients with autoimmune liver disease than in general HCC patients (Table 4).

Among both the AIH and PBC patient groups,

Table 4 Serum AFP levels, tumor sizes and number of HCC loci of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers (%)			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/ General-HCC patients	PBC-series/ General-HCC patients	AIH-series/PBC-series
Serum-AFP	Total: 37	Total: 47	Total: 15831			
AFP – (< 15 ng/mL)	15 (40.5)	13 (27.7)	5756 (36.4)	$P = 0.597$	$P = 0.216$	$P = 0.214$
AFP + (⩾ 15 ng/mL)	22 (59.5)	34 (72.3)	10075 (63.6)			
Serum-AFP (ng/mL)	Total: 37	Total: 47	Total: 15831			
(mean ± SD)	(2340.21 ± 8823.45)	(854.18 ± 2263.83)	NA			
< 15	15 (40.5)	13 (27.6)	5756 (36.4)			
15-199	12 (32.5)	16 (34.0)	5786 (36.5)	NA	NA	$P = 0.106$
200-399	1 (2.7)	6 (12.8)	902 (5.7)			($P < 0.001^b$)
400-999	3 (8.1)	2 (4.3)	907 (5.7)			
⩾ 1000	6 (16.2)	10 (21.3)	2480 (15.7)			
Maximum tumor size	Total: 36	Total: 48	Total: 15788			
of HCC				NA	NA	$P = 0.744$
(mean ± SD) (cm)	(3.75 ± 2.42)	(3.51 ± 1.69)	NA			($P = 0.028^a$)
< 2	14 (38.9)	11 (22.9)	5123 (32.4)			
2.1-5.0	16 (44.4)	29 (60.4)	7434 (47.1)			
⩾ 5.1	6 (16.7)	8 (16.7)	3231 (20.5)			
Number of HCC loci	Total: 38	Total: 49	Total: 16187			
(mean ± SD)	(1.58 ± 2.05)	(1.74 ± 1.97)	NA	NA	NA	$P = 0.418$
Single	33 (86.8)	38 (77.6)	9365 (57.9)			($P = 0.805$)
Double	2 (5.3)	7 (14.3)	2850 (17.6)			
Multiple	3 (7.9)	4 (8.1)	3972 (24.5)			

The *P*-value in the first row was calculated from the χ^2 test and Fisher's exact test. The *P*-value in the following row was calculated from the Mann-Whitney *U*-test and the *P*-value below, indicated in parentheses, was calculated from the *F*-test. $^aP < 0.05$, $^bP < 0.01$, Statistically significant. HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; AFP: α-fetoprotein; SD: Standard deviation; NA: Not available.

the most commonly selected forms of treatment were chemolipiodolization (CL) and transcatheter arterial embolization (TAE); other options included percutaneous ethanol injection therapy (PEIT) and radiofrequency ablation (RFA). Differences in the choice of therapeutic procedures were noted as follows, although no comparisons reached statistical significance following the Bonferroni correction: (1) The rate of CL or TAE among HCC patients with AIH was greater than the rate of operations among general HCC patients ($P = 0.048$), (2) The rate of CL or TAE among HCC patients with AIH was greater than the rate of PEIT and RFA among general HCC patients ($P = 0.018$), and (3) The rate of CL or TAE in HCC patients with PBC was greater than the rate of PEIT and RFA among general HCC patients ($P = 0.027$). Additionally, the frequency with which HCC patients with PBC chose to forgo treatment was significantly higher than the frequency with which general HCC patients chose to undergo PEIT or RFA ($P = 0.003$). Although not statistically significant, the frequency with which HCC patients with AIH refused therapeutic interventions was also higher than the frequency of PEIT or RFA in the general HCC population ($P = 0.038$, Table 5). Ideally, data on survival by treatment modality should be presented. However, the number of patients receiving each treatment modality who were able to be followed up to death was small. Hence, the mean period from HCC development to death was calculated from patient survival following all treatment options. Future prospective studies are needed to further analyze mean survival for each treatment alternative.

Across all three patient groups, we found that liver failure was the leading cause of death, followed by rupture of HCC. Among general HCC patients, neoplastic death was most common (1487/2700, 55.1%), although differences between causes of death did not reach statistical significance. Comparisons between patient groups showed that: (1) The rate of neoplastic death in general HCC patients was higher than the rate of variceal rupture in HCC patients with AIH ($P = 0.050$), (2) The rate of neoplastic death in general HCC patients was higher than the rate of gastrointestinal bleeding in HCC patients with AIH ($P = 0.013$), and (3) The rate of neoplastic death in general HCC patients was greater than the rate of variceal rupture in HCC patients with PBC ($P = 0.050$, Table 5).

DISCUSSION

While autoimmune liver disease is more common among women than men in Japan, HCC in our group of patients with autoimmune liver disease was more common in men than women (Table 2). Men with AIH had a 1.6-fold greater risk of HCC than women, while men with PBC had a 3.2-fold greater risk of HCC than women with PBC. Moreover, when we followed AIH and PBC patients during HCC surveillance, we noted that the rate of HCC development was higher in male patients with autoimmune liver disease than in female patients with autoimmune liver disease.

Cirrhosis was found in only 18/31 (58.1%) of HCC

Table 5 Therapy and outcome of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers (%)			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/ General-HCC patients	PBC-series/ General-HCC patients	AIH-series/ PBC-series
Therapy choices for HCC	Total: 38	Total: 47	Total: 17005	Operation *vs* CL or TAE $P = 0.048$[1]	RFA, PEIT *vs* CL or TAE	
CL or TAE	18 (47.4)	17 (36.2)	4636 (27.2)	RFA,PEIT,MCT *vs*	$P = 0.027$[1]	
Operation	8 (21.0)	16 (34.0)	5268 (31.0)	CL or TAE	RFA, PEIT *vs*	
RFA, PEIT, MCT	6 (15.8)	6 (12.8)	4890 (28.8)	$P = 0.018$[1]	No therapy	
Chemotherapy	0 (0)	0 (0)	765 (4.5)	RFA,PEIT,MCT *vs*	$P = 0.003$[2]	
Others	0 (0)	0 (0)	122 (0.7)	No therapy		
No therapy	6 (15.8)	8 (17.0)	1324 (7.8)	$P = 0.038$[1]		
Clinical outcome	Total: 37	Total: 49	Total: 16646			
Alive	20 (54.1)	31 (63.3)	13946 (83.8)	$P < 0.001$[b]	$P < 0.001$[b]	$P = 0.389$
Dead	17 (45.9)	18 (36.7)	2700 (16.2)			
Cause of death	Total: 16	Total: 18	Total: 2700	Neoplastic death	Neoplastic death	
Liver failure	8 (50.0)	8 (44.4)	581 (21.5)	*vs* variceal rupture	*vs* variceal rupture	
HCC rupture	3 (18.8)	4 (22.2)	172 (6.4)	$P = 0.050$[1]	$P = 0.050$[1]	
Variceal rupture	1 (6.2)	1 (5.6)	85 (3.1)	Cancer death *vs* GI		
GI bleeding	1 (6.2)	2 (11.1)	55 (2.0)	bleeding		
Neoplastic death	0 (0)	0 (0)	1487 (55.1)	$P = 0.013$[1]		
Others	3 (18.8)	3 (16.7)	320 (11.9)			

[b]$P < 0.01$, Statistically significant. [1]The calculated *P*-values did not reach statistical significance with Bonferroni correction; without the correction, however, *P*-values were below 0.05. [2]The calculated *P*-values reached statistical significance with Bonferroni correction. HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; CL: Chemolipiodolization; TAE: Transcatheter arterial embolization; RFA: Radiofrequency ablation therapy; PEIT: Percutaneous ethanol injection therapy; MCT: Microwave coagulation therapy; GI: Gastrointestinal.

patients with AIH, 29/44 (65.9%) of HCC patients with PBC, and in only 2250/4941 (45.5%) of the general Japanese HCC population. We did not add cases with liver fibrosis (LF) to the incidence of liver cirrhosis (LC) in the general Japanese HCC population, which may be one of the reasons why the incidence of liver cirrhosis was surprisingly low. Additionally, we think that the HCC cases with PBC and AIH and with non-cirrhotic liver, in which sufficient examinations and successful treatments were performed because of their higher hepatic reserve, were likely to be reported and submitted for publication. The possibility of bias in the selection of the reported cases should be raised.

Another interesting finding was that the interval between HCC diagnosis and death was shorter for patients with autoimmune liver disease than for the general HCC population of Japan[15]. Furthermore, although we found that serum AFP level did not vary widely, the maximum tumor size and number of HCC loci were considerably lower in patients with autoimmune liver disease than in general HCC patients (Table 4). One explanation for this finding may be a selection bias, as cases which were detected earlier and treated successfully were more likely to be submitted for publication. Despite a smaller tumor size and a lower number of HCC loci in patients with HCC arising in the setting of autoimmune liver disease at the time of HCC diagnosis, a shorter reported survival was not attributed to late detection of HCC and failure to survey patients with autoimmune liver disease for HCC, but was more likely to be due to advanced liver disease and cirrhosis. Future prospective studies will be needed to verify or refute these findings.

Although CL and TAE were the most frequently selected treatment modalities across all patient groups (Table 5), many patients ultimately refused treatment due to advanced age or social circumstances. Medical treatments using CL or TAE may be common because HCC cases are often inoperable due to cirrhotic liver disease in these patients. While survival may be related to the choice of therapeutic options, inconsistencies in data reporting over multiple decades and across multiple medical centers made the calculation of survival data difficult.

Several mechanisms explaining the development of HCC from autoimmune liver diseases have been proposed: enhanced progression to cirrhosis through progressive autoimmune hepatitis, decreased antitumor immune responses caused by long-term administration of steroids and immunosuppressants, or virus-mediated hepatitis[16,17]. In this study, we found significantly higher rates of HBV and HCV among PBC patients with HCC than among AIH patients with HCC. This finding may be attributable to the higher rates of blood transfusion in HCC patients with PBC ($P = 0.041$, Table 3). This result is supported by the findings of Shimizu *et al*[18], who reported that 3/16 (19%) HCC patients with PBC tested positive for prior HBV and present HCV infections. Given the high rates of prior HBV infections among HCC patients with PBC, it is possible that prior HBV infection predisposes patients to HCC through HBV-DNA becoming integrated into hepatocyte DNA. It has been reported that even in patients who test negative for serum HCV-RNA and serum HBV-DNA (less

than the sensitivity of HBV-DNA), liver tissue samples frequently test positive for HCV-RNA or HBV-DNA. This suggests a possible role for positive HCV-RNA or HBV-DNA in hepatic tissue in the development of HCC[19-23]. In the present study, however, only six HCC patients with AIH and two HCC patients with PBC were found to have detectable HBV-DNA and HCV-RNA in liver tissue samples. Aggressive liver biopsies should be taken to allow genetic analysis for HBV and HCV in liver tissue, in order to further study HCC cases with non-B, non-C hepatitis.

We reported high rates of HBV or HCV infection among HCC patients with PBC (Table 3); however, this is less surprising because international diagnostic criteria for AIH allocate negative points for positive HBV or HCV diagnostic tests[14]. Furthermore there are no definitive histological features that allow a clinician or pathologist to distinguish AIH from chronic viral hepatitis. Thus, HBV or HCV infected patients are rarely classified as having AIH in the modern era. In contrast, the unique histological features of PBC and the relative specificity of AMA tests allow clinicians to diagnosis and report cases of concurrent PBC and chronic viral hepatitis with a greater degree of confidence.

It has been reported that HCC develops significantly more often in patients with concurrent PBC and HCV infection than in patients with AMA-positive PBC[9]. The incidence of HCC associated with PBC has been suggested to have increased recently due to prolonged periods of liver cirrhosis resulting from longer survival on steroid therapy, concurrence of the hepatitis virus or alcohol intake with HCC, and the administration of immunosuppressants which may disturb immunoregulatory function[24,25]. In a proportional hazards analysis of patients with PBC, Shibuya *et al*[11] found 3 factors to be independently associated with the development of HCC: age at time of diagnosis, male gender, and a history of blood transfusion. Our findings showed that HCC cases arising from PBC were more common in men and those with liver cirrhosis.

While the number of HCC cases arising from PBC is stated to be small, it has been reported that the calculated crude incidence of HCC was 492.4/100 000 person years, and that HCC has a relatively high prevalence in PBC[7]. Furthermore, there is a dramatically increased risk for development of hepatobiliary malignancies in patients with PBC, with a relative risk of 46 ($P < 0.0001$) in women and 55 ($P < 0.0001$) in men[26].

Finally, several questions remain for the clinician. Namely, should AIH and PBC patients be screened for HCC? Should screening be limited to cirrhosis? Does the clinical course after diagnosis differ from other HCC patients? Late-stage AIH and PBC patients should be screened for HCC just as in HCV-related cirrhosis, given the similar reported incidence of HCC development in late-stage PBC[13]. Furthermore, Suzuki *et al*[27], reported very recently that patients of older age, male sex, history of blood transfusion, and any signs of portal hypertension or cirrhosis should be considered for HCC screening. A prospective study or a case control study for AIH patients is needed similar to that conducted for PBC patients.

At present, HCC transformation in early-stage pre-cirrhotic AIH and PBC were thought to be very rare. However, a high incidence of HCC development was observed in AIH and PBC patients with overlapping HCV and HBV infection, including occult HBV infection[9,19,20]. These patients should be closely followed using ultrasonography, CT-scanning and MRI of the abdomen, as well as tumor markers for HCC. Reports of HCC cases arising from "pure" AIH and PBC (with no history of blood transfusion, excessive alcohol intake, immunosuppressant administration, and with negative HBV and HCV serotyping) are rare[2,27-31]. El-Serag *et al*[32], in a multivariate analysis reported that AIH itself is not significant; however, our study indicates that early-stage AIH and PBC patients also have the potential to develop HCC. We advocate that "pure" or "early-stage" AIH and PBC cases should also be regularly screened for HCC.

Our data also indicate that the clinical course after diagnosis of HCC with AIH and PBC differs from virus-associated HCC, although prospective studies are needed to confirm these results. Clinicians should note the common clinical features of HCC cases with AIH and PBC at diagnosis, treatment, and follow-up of these patients.

Lastly, our findings also beg the question of why HCC rupture is the second most common cause of death in both groups of patients examined. We have recently reported a pelioid-type HCC patient with PBC, who died from rupture of HCC[33]. A peliotic change was observed more frequency in large poorly-differentiated and encapsuled HCC[34], and the features of pelioid-type HCC were high blood flow into the HCC, high pressure in the tumor and fibrous capsular formation. It is unknown whether the ruptured HCCs in the present study had these features, as this study had severe limitations because it was retrospective. Tumors in such patients may grow rapidly, and pathophysiological factors shared by both patient groups may trigger the rupture of HCC. A prospective study on the cause of death and a pathologic study of ruptured HCC with AIH and PBC is awaited with great interest.

Further clinical and laboratory studies are needed to describe which pathological, biological and genetic features are common among HCC cases arising from AIH and PBC. How HCC in these patients relates to viral hepatitis also requires further clarification. The present study was retrospective; however, this is the first study to date that highlights the importance of these future research topics. Future prospective studies on these important subjects are required.

COMMENTS

Background

Hepatocellular carcinoma (HCC) development in autoimmune hepatitis (AIH) as well as in primary biliary cirrhosis (PBC) is a rare event. The common clinical features of HCC associated with AIH and PBC have not yet been extensively

described.

Research frontiers

In this study, we characterized these common features through a systematic review of the literature conducted using the Japana Centra Revuo Medicina database. We demonstrated common clinical features among cases of HCC arising from AIH and PBC in Japan.

Innovations and breakthroughs

We found common clinical features in HCC cases with AIH and PBC as follows, (1) HCC was more common in men than in women with AIH or PBC. (2) Many patients underwent chemolipiodolization (CL) or transcatheter arterial embolization (TAE). (3) Liver failure was the primary cause of death among patients in this study, followed by tumor rupture. (4) The survival interval between diagnosis and death was fairly short.

Applications

The present study was retrospective; however, this is the first study to date that highlights the common clinical features in HCC cases with AIH and PBC. Future prospective studies of these important subjects are required.

Peer review

This is a systematic literature review of HCC cases with AIH and PBC published throughout Japan. The review is clearly written and highlights a very interesting topic.

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BRIEF ARTICLES

Interaction of hepatitis C virus envelope glycoprotein E2 with the large extracellular loop of *tupaia* CD81

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Abstract

AIM: To further analyze the interaction of *tupaia* CD81 with hepatitis C virus (HCV) envelope protein E2.

METHODS: A *tupaia* CD81 large extracellular loop (CD81 LEL), which binds to HCV E2 protein, was cloned and expressed as a GST-fusion protein, and interaction of HCV E2 protein with a *tupaia* CD81 LEL was evaluated by enzyme-linked immunosorbent assay (EIA).

RESULTS: Although *tupaia* and human CD81 LEL differed in 6 amino acid changes, *tupaia* CD81 LEL was strongly recognized by anti-CD81 antibodies against human CD81 LEL conformation-dependent epitopes. Investigating LEL CD81-E2 interactions by EIA, we demonstrated that binding of *tupaia* CD81 LEL GST fusion protein to recombinant HCV E2 protein was markedly reduced compared to binding of human CD81 LEL GST fusion protein to recombinant HCV E2 protein.

CONCLUSION: These data suggest that the structural differences in-between the *tupaia* and human CD81 may alter the interaction of the large extracellular loop with HCV envelope glycoprotein E2. These findings may be important for the understanding of the mechanisms of binding and entry of HCV to PTHs.

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Key words: Hepatitis C virus E2 protein; Tupaia; CD81, Bind; Enzyme-linked immunosorbent assay

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INTRODUCTION

Hepatitis C virus (HCV) is a major cause for posttransfusion and community-acquired hepatitis worldwide[1-4]. The majority of HCV-infected individuals develop chronic hepatitis that may progress to liver cirrhosis and hepatocellular carcinoma (HCC)[5]. Virion contains an approximately 9.5 kb long positive-strand RNA genome encoding for a single polyprotein containing 3010-3030 amino acids[6-8]. The polyprotein is cleaved co- and post-translationally by host cellular and viral proteases into at least ten different products. The predicted structural components of the virus comprise the core and two envelope glycoproteins: E1 and E2. The E2 protein is responsible for initiating viral attachment to receptor(s) on potential host cells due to its ability to bind to human cells[6,9]. CD81 has first been identified as a HCV E2 binding molecule by expression cloning by Pileri and colleagues[10]. The E2 binding site of CD81 is located within the LEL domain[10-13]. Further studies then demonstrated a key role of CD81 as a host entry factor for infection of human hepatoma cells with recombinant HCV pseudoparticle and tissue-culture-derived HCV (HCVcc)[14-18]. Furthermore, one very recent study has demonstrated evidence that CD81 is also involved in HCV infection of primary human hepatocytes using serum-derived HCV[19]. However, there appear to be subtle differences regarding the role

of CD81 for productive infection of human hepatoma cells and human hepatocytes using serum-derived or recombinant virus[19-24].

Recently, a novel *in vitro* cell culture model system has been established for HCV with primary tupaia hepatocytes (PTHs)[25-27]. Using this cell culture system, we found that HCV E2 protein binding to PTHs and infection of PTHs with HCV could not be blocked with soluble CD81 and anti-CD81[25]. To further investigate the role of CD81 in the infection of PTHs with HCV, we cloned a tupaia CD81 large extracellular loop (LEL) and analyzed the interaction of *tupaia* CD81 with HCV E2 protein *in vitro*. The results indicate that changes occur in 6 amino acid residues of *tupaia* CD81 LEL and the ability of *tupaia* CD81 LEL to bind to HCV E2 is significantly decreased compared with human CD81 LEL.

MATERIALS AND METHODS

Reagents

Recombinant HCV E2 protein, and mouse anti-HCV E2 (3ES) were generously provided by M Houghton (Chiron Corp., Emeryville, CA). Human CD81 LEL (wild type and T163A mutant) and AGM CD81 LEL protein were kindly provided by Dr. S Levy (Department of Medicine, Stanford Medical School, Palo Alto, CA)[12]. Mouse monoclonal anti-CD81 antibodies 5A6 and 1D6 have been described elsewhere[11,12,28]. Glutathione S-transferase (GST) expression vector, PGEX-2T, was purchased from Amersham Pharmacia Bioteh Inc.

Cloning of tupaia CD81 LEL

Total cellular RNA was isolated from PTHs with RNeasy kits (Qiagen, GmbH, Hilden, Germany) according its manufacturer's instructions. cDNA was transcribed from cellular RNA using rTth reverse transcriptase. *Tupaia* CD81 (TupCD81) LEL was amplified by PCR using Pfu DNA polymerase (Stratagene) and human CD81-specific primers covering the LEL domain (sense: 5'-TAACGG ATCCAACAAGGACCAGATTGCCAAGGA-3', anti-sense: TAACGAATTCACAGCTTCCCGGAGAAGAG CTC-3'). The PCR products were then cloned into pCR-Blunt Ⅱ-TOPO (Invitrogen, Groningen, Netherlands). An EcoR I /BamH I fragment was subcloned into pGEX-2T (Amersham Pharmacia Biotech Inc.) with the same enzyme digestion. The resulting plasmid (named p2T-TupCD81 LEL) was sequenced using CD81-specific primers. Plasmids expressing African green monkey, and human CD81 LEL (both wild type and genetically modified T163A type) were kindly provided by Dr. S Levy and have been described previously.

Expression and purification of glutathione S-transferase (GST)-CD81 LEL fusion proteins

Expression and purification of GST-CD81 LEL fusion protein were performed as previously described[13]. Briefly, BL21 *E. coli* were transformed with plasmid DNA. Transfected *E. coli* were grown in 12 mL LB medium containing ampicillin (50 mg/L) at 30-32℃ until the optical density (OD) value of the culture medium reached 0.6-0.8 at *A*600. Protein expression was then induced by adding isopropyl β-D-thiogalactoside (IPTG, 0.75 mmol/L). After cells grew for additional 3 h, they were lysed by sonication and the fusion proteins were purified using the MicroSpin GST purification module (Amersham Pharmacia Biotech). GST protein expressed in control plasmid pGEX-2T was purified in parallel. The purified proteins were analyzed using SDS-PAGE under non-reducing conditions, quantified, aliquoted, and stored at -80℃ until use.

Test of HCV E2-CD81 LEL interaction

A 96-well microtiter plate was coated with GST-CD81 fusion protein (5 mg/L) at 4℃ overnight. After washing 5 times with PBS and blocking with 4% dry milk (in PBS) at room temperature for 1 h, 50 μL of recombinant HCV E2 protein (with reciprocal dilutions, starting from 2 mg/L) was allowed to bind to CD81 LEL at 4℃ overnight. The bound E2 was detected using anti-E2 (3E5; diluted at 1:2000 in PBS containing 1% Tween 20 and 1% BSA) and HRP-conjugated anti-mouse IgG (diluted at 1:5000 in PBS containing 1% Tween 20 and 1% BSA). GST protein and human and AGM CD81 LEL were tested as controls. For GST test, goat anti-GST (in dilution starting at 1:1000) (Amersham Pharmacia Biotech) was added, and incubated at room temperature for 1 h. The bound anti-GST was detected using HRP-conjugated anti-goat IgG. The bound HRP-conjugated second antibodies were quantified by colorimetric reaction with an Abbott OPD reagent kit (Abbott Laboratories, North Chicago, IL), and the OD value was measured at 490 nm on a Bio-Rad plate reader (Bio-Rad, Hercules, CA).

RESULTS

Tupaia CD81 LEL amino acid sequence

It has been demonstrated that the E2 binding site of CD81 is located within the LEL domain[10,12,13]. To study the E2-CD81 LEL interaction *in vitro*, *tupaia* CD81 LEL was cloned and sequenced. The deduced amino acid sequence of *tupaia* CD81 LEL cDNA showed mutations in 6 amino acid residues when compared with human CD81 LEL as previously showed[25]. These mutations were clustered around the E2 binding head subdomain (Figure 1), according to the 3D structure of human CD81 LEL[29].

Analysis of tupaia CD81 LEL-E2 interaction in vitro

To analyze the interaction between HCV glycoprotein E2 and *tupaia* CD81 LEL *in vitro*, *tupaia* CD81 LEL was expressed as a GST-fusion protein in *E. coli*. The correct expression and folding of *in vitro*-synthesized *tupaia* CD81 LEL were confirmed by analyzing the purified protein with non-reducing SDS-PAGE and immunoblot using anti-CD81 antibodies against conformation-dependent epitopes. As shown in Figure 2A and B, *tupaia* CD81 LEL was recognized by defined anti-human CD81

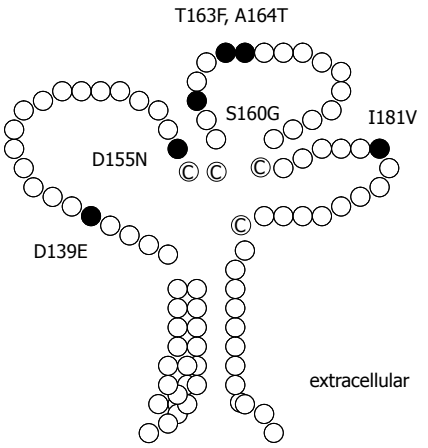

Figure 1 Putative structure of *tupaia* CD81. *Tupaia* CD81 LEL cDNA was cloned and sequenced as described in Materials and Methods. Deduced amino acid sequence was compared with that of human. The *tupaia* CD81 secondary structure was drawn according to the three-dimensional structure of human CD81 LEL, and changes in six amino acids of *tupaia* CD81 LEL were illustrated by black color.

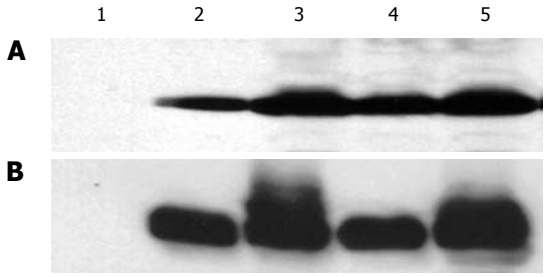

Figure 2 Expression and purification of soluble tupaia CD81 LEL using mouse monoclonal anti-human CD81 5A6 (A), and 1D6 (B) antibodies. *Tupaia* CD81 LEL was expressed and purified in *E. coli* as a GST fusion protein as described in Materials and Methods. Purified proteins were subjected to SDS-PAGE, and immunoblot using mouse monoclonal anti-human CD81 5A6 and 1D6 antibodies. Tup: *tupaia*; h: human; AGM: African green monkey; hCD81 LEL T163A: human CD81 containing a mutation of T to A at amino acid residue 163; 1: GST; 2: TupCD81 LEL-GST; 3: hCD81 LELT163A-GST; 4: AGMCD8 LEL-GST; 5: hCD81 LEL-GST.

antibodies (5A6 and 1D6), similar to human or African green monkey CD81 LEL. The data suggest that folding of CD81 LEL-GST fusion protein is comparable to that of the native molecule, while slight differences might exist in the 3D structure of human and *tupaia* CD81 LEL since the staining intensity of TupCD81 was week compared with that of human CD81 (both wild and T163A mutation type) using antibody to human CD81 conformation-dependent epitopes. Interaction of *tupaia* CD81 LEL with recombinant E2 protein was analyzed with EIA, and compared with that of human- or African green monkey-derived CD81 LEL. Binding of *tupaia* CD81 LEL to HCV E2 protein was markedly reduced compared with the native or mutant human CD81 LEL (Figure 3).

DISCUSSION

Recombinant HCV E2 could bind to *tupaia*, but not to rat hepatocytes in a dose-dependent manner and PTHs could be infected with HCV *in vitro*[25,26]. Furthermore,

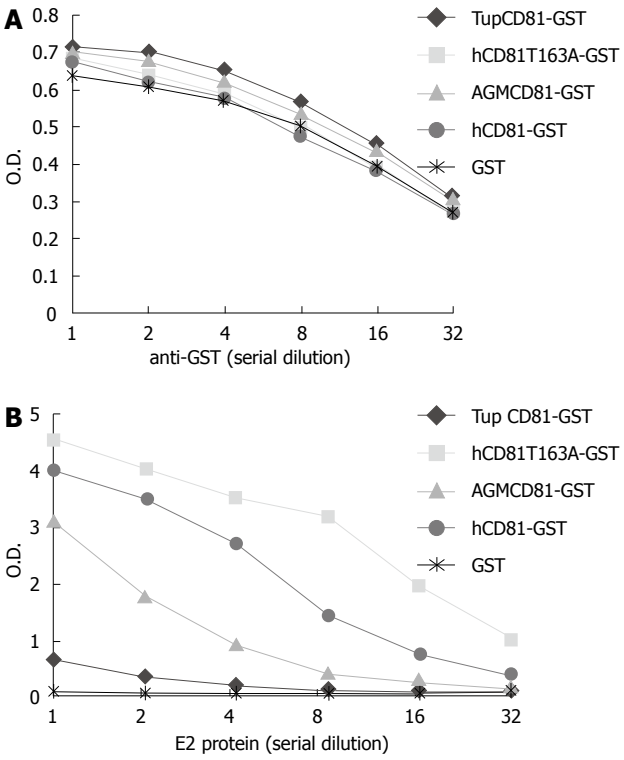

Figure 3 Interaction between *tupaia* CD81 LEL and HCV E2 protein. Plates were coated with 100 μL of recombinant GST or GST-CD81 LEL fusion proteins (5 mg/L), reciprocally diluted anti-GST antibody (A) (starting dilution at 1:1000) or HCV E2 protein (B) (starting concentration 2 mg/L) was added to the plates. Binding of anti-GST or HCV E2 protein was assessed as described in Materials and Methods. OD: optical density; Tup: tupaia; h: human; AGM: African green monkey; hCD81T163A: human CD81 containing a mutation of T to A at amino acid 165.

we demonstrated that both the binding of HCV E2 to PTHs, and infection of PTHs with HCV could not be blocked with soluble CD81 and anti-CD81 exhibiting the blocking ability of HCV E2 to bind to lymphoma cell lines, indicating that binding of E2 to PTHs and infection of *tupaia* hepatocytes with HCV might require additional or other molecules besides CD81. To further characterize the HCV E2-*tupaia* CD81 interaction, we cloned the HCV E2 binding domain of CD81, the CD81 LEL, and investigated the reaction of HCV E2 with *tupaia* CD81 *in vitro*.

CD81, a member of the super-family of tetraspanins, comprises 4 transmembrane (TM1-4) and two extracellular loops. The HCV E2 binding domain locates in the large extracellular loop (LEL), which folds to form a mushroom-like 3D structure, and is stabilized by a number of specific interactions within the defined amino acid residues[29]. In the present study, the deduced amino acid sequence of *tupaia* CD81 LEL showed changes in only 6 amino acid residues compared to human CD81 LEL, while all the residues necessary for the 3D structure-stabilization were conserved, suggesting that soluble *tupaia* CD81 LEL folds in a manner comparable to human CD81 LEL. This hypothesis was confirmed by the cross interaction of *tupaia* CD81 LEL with anti-human CD81 antibodies against conformation-dependent epitopes (Figure 2).

It has been reported that HCV E2 binds to the

head subdomain of CD81 LEL consisting of about 60 amino acid residues[29]. Distinct amino acid mutations can affect CD81-E2 interaction. African green monkey (AGM) CD81 LEL contains only 4 amino acid residues compared with human CD81 LEL, and shows reduced E2 binding[10-12]. Reduced E2-binding of AGM CD81 is due to a mutation of phenylalanine to leucine at the amino acid residue 186 (F186L)[12]. Interestingly, mutation of threonine to alanine at the amino acid residue 163 (T163A) can enhance E2-CD81 binding[12]. In this study, all the six mutant residues of *tupaia* CD81 LEL were clustered at its head subdomain which is the binding site for HCV E2, while the phenylalanine at residue 186, and the 4 cysteine residues, which are pivotal for human CD81-HCV E2 binding, were conserved in *tupaia* CD81. When purified *tupaia* CD81 LEL was tested for its binding to recombinant HCV E2 protein, only a mild E2-CD81 interaction was observed. By contrast, human CD81 LEL (both wild-type and genetically modified CD81 containing a T163A mutation) bound firmly to E2 protein. Interestingly, threonine at residue 163 of TupCD81 LEL changed into phenylalanine. The reduced E2 binding ability of *tupaia* CD81 was not due to the amount of protein coating the plates, since the GST activity of those fusion proteins was nearly similar. From the perspective of the 3D structure of CD81 LEL, mutations at amino acid residues 155 and 181 of *tupaia* CD81 LEL may be responsible for the reduced binding in *Tupaia* CD81 to HCV E2 protein[29]. The binding of HCV E2 to AGMCD81 LEL in this study different from a previous study[12]. This discrepancy might be due to the differences in the recombinant HCV E2 proteins. E2 used in this study is C-terminally truncated at amino acid 715, whereas other investigators used E2 C-terminally truncated at amino acid 661.

Although our studies were limited to evaluate CD81 LEL-E2 interaction with EIA, and need to be confirmed in model systems expressing full-length CD81 in transfected mammalian cell lines, the differences in HCV E2 binding between *tupaia* CD81 LEL and human CD81 LEL are consistent with our previous functional data assessing E2 binding to PTHs and HCV infection of PTHs in the presence of anti-CD81 antibodies. Taken together, these results indicate that although CD81 may play a functional role as a co-factor for entry of HCV into PTHs, it is likely that other or additional molecules besides CD81 play a key role in HCV entry into PTHs. These may include other identified HCV host factors including SR-BI[27] or Claudin-1[30]. Alternatively, other not yet identified host entry factors may mediate HCV-PTH interaction.

COMMENTS

Background

CD81, a member of the superfamily tetraspanins, comprises four transmembrane (TM1-4) and two extracellular loops. The HCV E2 binding domain locates in the large extracellular loop (LEL). The LEL folds to form a mushroom-like 3D structure, which is stabilized by a number of specific interactions within the defined amino acis residues.

Research frontiers

Hepatitis C virus (HCV) is a major cause for posttransfusion and community-acquired hepatitis worldwide. The majority of HCV-infected individuals develop chronic hepatitis that may progress to liver cirrhosis and hepatocellular carcinoma (HCC). The predicted structural components of HCV comprise the core and two envelope glycoproteins: E1 and E2. The E2 protein is responsible for initiating viral attachment to receptor(s) on potential host cells due to its ability to bind to human cells. HCV E2 could specifically bind to cell surface molecule CD81 expressed in lymphoma cells. Anti-HCV antibodies from chimpanzees that are protected against homologous HCV challenge by vaccination with envelope glycoproteins inhibit E2 binding to CD81, suggesting that CD81 represents a candidate receptor for HCV infection. Whether CD81-E2 interaction can mediate virion entry into host cells is unknown and has not been tested in a suitable model.

Innovations and breakthroughs

A novel *in vitro* cell culture model system has been established for HCV with primary tupaia hepatocytes (PTHs). Using this cell culture system, we found that HCV E2 protein binding to PTHs and infection of PTHs with HCV could not be blocked with soluble CD81 and anti-CD81. To further investigate the role of CD81 in the infection of PTHs with HCV, we cloned a tupaia CD81 large extracellular loop (LEL) and analyzed the interaction of *tupaia* CD81 with HCV E2 protein *in vitro*. The results indicate that changes occur in 6 amino acid residues of *tupaia* CD81 LEL and the ability of *tupaia* CD81 LE to bind to HCV E2 is significantly decreased compared with human CD81 LEL.

Applications

Tupaia CD81 has a reduced ability to bind to HCV E2 protein. HCV entry and infection of PTHs with HCV might occur through receptor(s) besides CD81.

Peer review

The manuscript is well written, but needs clarification of the importance of the found amino acid changes with regard to the interaction of *tupaia* CD81 and HCV E2 binding.

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CASE REPORT

Perigastric extraskeletal Ewing's sarcoma: A case report

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Abstract

Ewing's sarcoma (ES) is a neoplasm of undifferentiated small round cells, which occurs in the bones and deep soft tissues of children and adolescents. We present a rare case of a 44-year-old woman with gastric ES presenting with epigastric pain and weight loss. Ultrasound and computed tomography scans indicated a solid/cystic mass in the pancreatic tail. At laparotomy, the tumor was found attached to the posterior surface of the stomach, completely free from the pancreas, with no lymphadenopathy or local metastases. The polynodal, partly pseudocystic, dark-red soft tumor was excised. Histopathology revealed an anaplastic small-round-cell tumor with strong membranous CD99 immunoexpression. Additionally, there was patchy immunostaining for S-100 protein, vimentin, protein gene product (PGP) 9.5 and neuron-specific enolase, and weak focal CD117 cytoplasmic immunoreactivity. The patient had no adjuvant chemotherapy; her postoperative recovery was uneventful, and she remains symptom-free, and without any sign of recurrence at 20 mo. To the best of our knowledge, this is only the third ever case of gastric ES.

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Key words: Stomach; Extraskeletal; Ewing's sarcoma

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INTRODUCTION

Ewing's sarcoma, or primitive neuroectodermal tumour (ES/PNET), is a neoplasm of undifferentiated small round cells that occurs in children, adolescents and young adults[1,2]. Although predominantly affecting the bones and deep soft tissues[2], these sarcomas are also being described as affecting the visceral organs, with increasing frequency. These extraskeletal ES/PNET sarcomas are histologically indistinguishable from the bony type[3], and have been documented in the pancreas[4,5], vagina[6], rectovaginal septum[7], small bowel[8,9], prostate[10], ovaries[11], esophagus[12], and kidney[13]. Two cases of gastric ES/PNET have been previously reported: the first in a 14-year-old boy[14] and the second in a 66-year-old woman[2]. We describe the third ever case of gastric ES.

CASE REPORT

In January 2006, a 44-year-old obese woman presented with a 6-mo history of epigastric pain and weight loss of 5 kg. On examination, she had only mild tenderness in the epigastrium. All standard laboratory data were within normal limits, though fasting cholesterol was elevated at 6.80 μmol/L (normal range 3.1-6.4 μmol/L), and fasting triglyceride was 2.30 mmol/L (normal < 1.95 mmol/L); erythrocyte sedimentation rate was 14 mm/h. Ultrasonography (US) and computed tomography (CT) scans (Figure 1) confirmed a solid/cystic mass measuring 66 mm × 46 mm in the tail of the pancreas.

The patient underwent laparotomy for the tumor at the tail of the pancreas; but, at operation, the only pathology found within the abdomen was a mass along the posterior wall of the stomach (Figure 2). There was no lymphadenopathy in the vicinity of the tumor or the upper retroperitoneum. The tumor was dark-red in color, moderately soft, covered by a thin light-grey membrane,

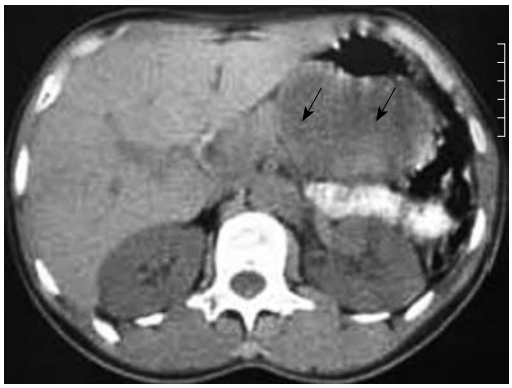

Figure 1 CT scan indicating a tumor at the tail of the pancreas (arrows).

Figure 2 Photograph showing the tumor on the posterior wall of the stomach.

Figure 3 Photograph showing a cross section of the tumor.

and adherent to the stomach along a surface of 3 cm × 3 cm. Intraoperative frozen section biopsy failed to clarify either the tumor type or the eventual malignant potential. Thus, when the tumor was completely excised, and no significant damage was seen on the stomach wall, the decision was made to not proceed to a gastric resection.

The pathological specimen measured 10 cm in its greatest diameter. On gross inspection, the cut surface showed large central hemorrhagic and necrotic changes and pseudocystic degeneration. The tumor tissue was largely light grey and solid, with some softer and more friable, reddish congested areas (Figure 3). Microscopic examination revealed a hypercellular diffuse, and rather monotonous proliferation of small round cells with clear

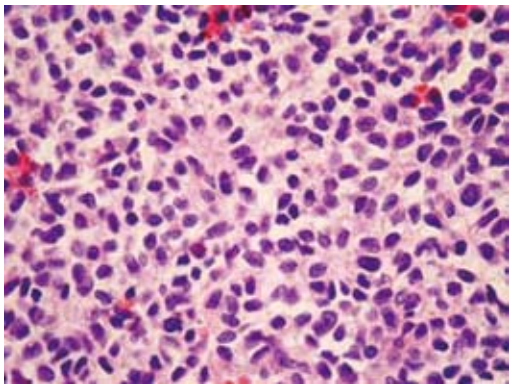

Figure 4 Histological appearance of the diffuse neoplastic infiltration showing rather uniform diffuse small round cells and abortive pseudorosette formation (HE, x 112).

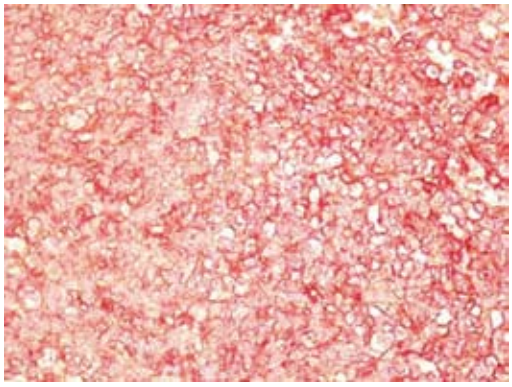

Figure 5 Tumor cells showing strong diffuse membrane immunohistochemical reactivity with CD99 antibodies [labeled streptavidin biotin (LSAB+) method, 3-amino-9-ethyl carbazole (AEC) visualization, x 112].

cytoplasm and relative nuclear uniformity. However, there were also areas showing nuclear atypia and abortive pseudorosette formation (Figure 4). Microcystic and hemorrhagic changes were seen on most of the sections, as well as sharply demarcated borders frequently covered by intact serosa. Tumor necrosis was minimal, although atypical mitotic figures were seen with a mitotic index of 14 per 50 high power fields.

Immunohistochemical examination with monoclonal antibodies excluded most of the differential diagnoses. Immunostaining was negative for pancytokeratin AE1/AE3, epithelial membrane antigen (EMA), leukocyte common antigen, chromogranin A, synaptophysin, desmin, muscle-specific antigen, the α subunit of smooth muscle antigen (SMA), glial fibrilar acidic protein, neurofilament protein, α-inhibin, melanin A and CD34. The diagnosis of ES/PNET was established by the presence of strong membranous CD99 immunoexpression by the vast majority of tumor cells (Figure 5), in addition to clear, but patchy S-100 protein, vimentin, protein gene product (PGP) 9.5 and neuron-specific enolase (NSE) immunostaining, as well as weak focal CD117 cytoplasmic immunoreactivity in very few neoplastic cells. Fluorescent *in situ* hybridization (FISH) was not available to us.

The patient did not receive any adjuvant chemotherapy or radiotherapy; her postoperative recovery was unevent-

ful, and she remains symptom-free and without recurrence on US or CT scan 20 mo later.

DISCUSSION

Histologically, ES/PNET is composed of small round cells that are usually rich in glycogen, and the neuroepithelial morphologic differentiation is confirmed by pseudorosette formation. Immunohistochemically, the neuroendocrine phenotype is confirmed by positivity to CD99, and to NSE and S-100 to a lesser extent, as these are also found in a number of other small-round-cell tumors[2]. FISH testing for the presence of the t (11;12) translocation can be particularly useful when the tumor occurs in older patients or in an unusual site[2,15], as well as to differentiate from desmoplastic small round cell tumors (DSRCT)[15]. The ultimate diagnosis should be based on both histology and immunohistochemistry[16].

We considered several other small-round-cell tumors in our differential diagnoses. Mesenchymal chondrosarcoma, and small-cell osteosarcoma were excluded as no chondroid or osteoid differentiation was found. Embryonic rhabdomyosarcoma was excluded as all muscular markers were negative. Intra-abdominal desmoplastic round-cell tumor was excluded as cytokeratin and EMA markers were negative, and nodular dissemination was absent. Hemangiopericytoma, glomus and other perivascular tumors were excluded on the basis of CD34 and SMA negativity.

The results of surgery alone for extraskeletal ES are poor in most cases, while patients receiving multimodal chemotherapy and radiotherapy have a much better prognosis[17]. Through the combination of local surgical treatment and systemic chemotherapy, long-term survival has improved from 10% to 50%-60% or greater[18,19], although the pathologist and oncologist will need to decide whether treatment regimens for tumors are better based on their phenotype or their genotype, when these two profiles are seemingly in conflict[16].

The two previously published cases of ES of the stomach both had poor outcomes. A 14-year-old boy with gastric ES was also found to have a diffuse metastatic lesion in the liver. He underwent a subtotal gastrectomy and lymphadenectomy followed by chemotherapy with a tyrosine kinase inhibitor because of intense expression of CD117 (c-kit), but died[14]. A 66-year-old woman with ES within the antropyloric area of the stomach underwent a distal gastrectomy and lymphadenectomy, but despite adjuvant chemotherapy, she also died 10 mo postoperatively[2].

Following postoperative pathological analysis, our patient was presented to the oncologists, who decided not to give any adjuvant chemotherapy. Despite this, she remains clinically well, and without recurrence to the present day (20 mo later).

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CASE REPORT

A case report of endocrine cell carcinoma in the sigmoid colon with inferior mesenteric vein tumor embolism

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Abstract

We report a case of endocrine cell carcinoma in the sigmoid colon with inferior mesenteric vein (IMV) tumor embolism. A 79-year-old woman was admitted to our hospital with narrowing of the stools. We performed colonoscopy, computed tomography and positron emission tomography, which disclosed sigmoid colon cancer with IMV tumor embolism. She underwent sigmoidectomy and lymph node dissection. The tumor was diagnosed as endocrine cell carcinoma (type 4, pSS, med, INFα, v3, n1, stage Ⅲb). Immunohistochemically, chromographin A, synaptophysin, cytokeratin 20 and mucicarmine showed partial staining, and CD56 was totally reactive. Three months after operation multiple liver metastases appeared. She was treated with chemotherapy of cisplatin (CDDP) + irinotecan (CPT11). This case highlights the aggressiveness of endocrine cell carcinoma with tumor embolism, and it is essential to establish an accurate diagnosis and effective treatment.

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Key words: Enteroendocrine cells; Tumor embolism; Carcinoid tumor; Colon cancer, Cisplatin; Irinotecan

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INTRODUCTION

Endocrine cell carcinoma of the colon and rectum is uncommon, and accounts for less than 1% of colorectal cancer[1]. It is well known that colorectal carcinoma of common pathology has a relatively good prognosis, whereas endocrine cell carcinoma of the colon and rectum has a very poor prognosis. Many patients with endocrine cell carcinoma have liver and lymph node involvement at the time of diagnosis.

Tumor embolism is an uncommon complication in digestive system cancers. The majority of cases of tumor embolism are related to renal carcinoma and liver carcinoma, not colon and rectum carcinoma. In fact, there has never been a report of endocrine cell carcinoma of the colon and rectum complicated by tumor embolism. Treatment of endocrine cell carcinomas of the colon and rectum are very difficult. We, herein, report the malignant potential, and the treatment of colorectal endocrine cell carcinoma with tumor embolism of the inferior mesenteric vein (IMV).

CASE REPORT

A 79-year-old woman experienced narrowing of the stools that lasted for more than a few days. She had been well since she complained of intermittent abdominal pain. She had no remarkable past medical history. Laboratory findings showed no abnormalities. Colonoscopy disclosed a circumferential type 2 lesion in the sigmoid colon (Figure 1). However, biopsy of this tumor revealed no malignant lesion, only regenerative colon mucosa. Abdominal computed tomography (CT) and positron emission tomography (PET) showed hypertrophy of the sigmoid colon wall with fusiform enlargement of the IMV extending up to the confluence with the splenic vein and a few lymph node metastases in the mesocolon (Figure 2). There were no liver metastases. These findings suggested that she had sigmoid colon cancer (cT3N2M0 Stage ⅢB) with IMV tumor embolism. Considering the possibility of progressive cancer obstruction and tumor embolism, we decided to perform radical sigmoidectomy and lymph node dissection without preoperative chemotherapy. Perioperatively, we paid special attention to make an incision in the IMV first before the tumor was isolated. The specimen of tumor was a 11.5 cm × 3.5 cm, type 4 cancer (Figure 3A), pSS, med, INFα, v3, PM0,

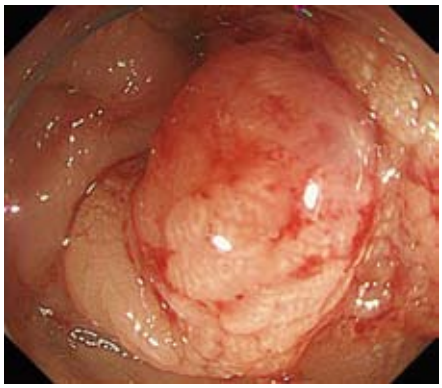

Figure 1 Colonoscopy showing a type 2 shaped tumor mainly located in the sigmoid colon.

Figure 2 Computed tomography showing a hypertrophic colon wall in the sigmoid colon and dilation of IMV (arrows).

DM0, RM0, DX with tumor embolism in the IMV, and the clinical stage was stage ⅢB pT3N1M0 in the UICC TNM classification. The tumor embolism in the IMV was a solid lesion 14 cm long and 2 cm across in transverse section (Figure 3A and B). Microscopically, it was seen that the tumor had invaded the vein wall and expanded into the extra space (Figure 4).

Histological features included an irregular pattern of the nuclei in size and mitotis (Figure 5). The tumor was diagnosed pathologically as an endocrine cell carcinoma histochemically, using CD56, chromographin A, synaptophysin, cytokeratin 7, cytokeratin 20 and mucicarmine. Chromographin A, synaptophysin, cytokeratin 20 and mucicarmine were partly stained and CD56 was totally reactive (Figure 6).

The postoperative course was mostly uneventful. However, three months after operation, multiple liver metastasis appeared. Now, she is being treated with chemotherapy of cisplatin (CDDP) + irinotecan (CPT11) and still alive after 14 mo.

DISCUSSION

This case is the first report of a sigmoid colon endocrine cell carcinoma invading into IMV as a tumor embolism. Endocrine cell carcinomas of the colon and rectum are uncommon, comprising less than 1% of colon and rectal cancer[1]. Endocrine cell carcinomas do not include carcinoid tumors that have a benign course compared to adenocarcinoma and endocrine cell carcinoma. Iwafuchi *et al* have proposed four classifications of endocrine

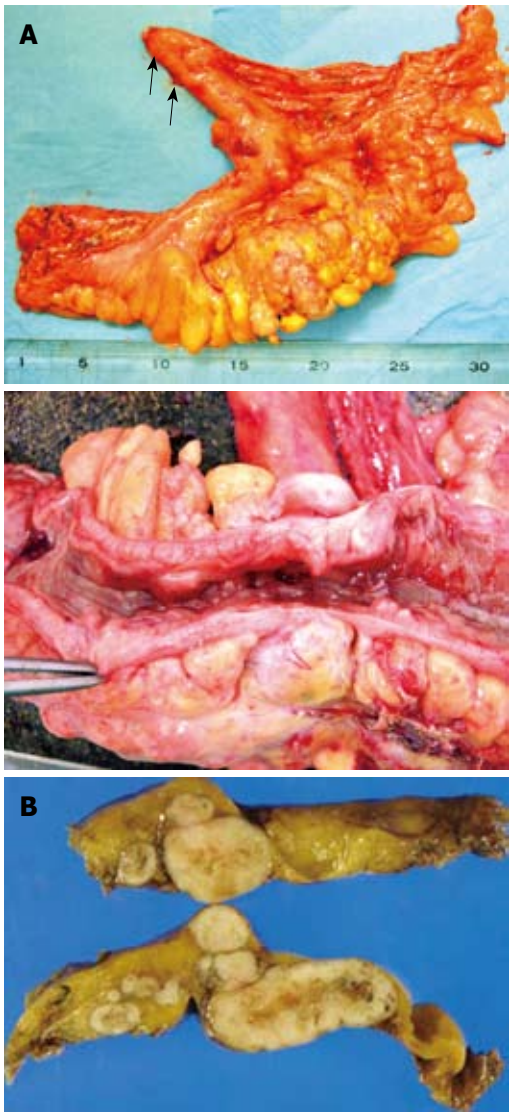

Figure 3 Resected specimen. A: A type 2 shaped tumor was located in the sigmoid colon with tumor embolism of IMV which was 14 cm long (arrows); B: In transverse section, the tumor embolism was 2 cm long.

cell carcinoma derived from: (1) preexisting general-histological adenocarcinomas; (2) preexisting carcinoids; (3) nonneoplastic multipotential stem cells and (4) nonplastic immature endocrine cells[2]. Recently, it has been thought that endocrine cell carcinomas predominantly arise from endocrine precursor cell clones occurring in preexisting adenocarcinoma components, transforming into endocrine cell carcinomas during rapid clonal expansion under the influence of *p53* gene alteration[3].

From 1986 to 2007, a total of 87 cases of endocrine cell carcinomas of the colon and rectum were reported in Japan (Table 1). The average patient age was 60.8 years (range, 34-89 years). There were 44 males and 43 females. Tumors were located as follows: 2 in appendix, 9 in cecum, 17 in ascending colon, 9 in transverse colon, 2 in descending colon, 2 in sigmoid colon and 47 in rectum. It suggests that endocrine cell carcinomas arise in any part of the colon, but particularly in rectum. The lesions invaded into subserosa or pericolic tissue (51/66; 77%). The rate of vascular invasion (32/44; 72%) was also very high. Endocrine cell carcinomas are aggressive

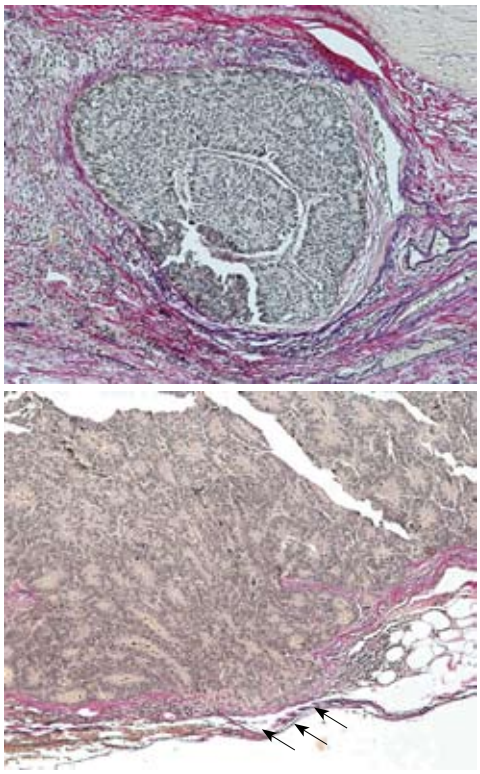

Figure 4 Histological features. Nuclei of the endocrine cell carcinoma cells were irregular in size, and mitotis was frequently seen with extensive vein invasion partially penetrating the vein wall (arrows).

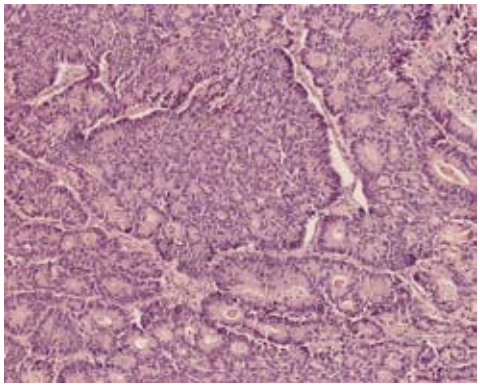

Figure 5 Histological features. Nuclei of the endocrine carcinoma cells were irregular in size, and mitosis was frequently identified.

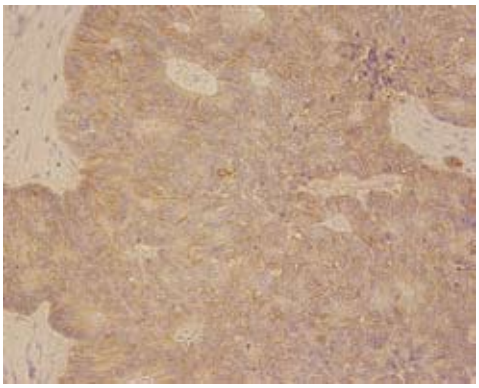

Figure 6 Immunohistochemical features. The endocrine carcinoma cells were immunoreactive for chromographin A, synaptophysin and CD56 (NCAM).

Table 1 Review of the literature

Clinical features of 87 patients with endocrine cell carcinoma	
Characteristic	
Sex M/F (n = 87)	44/43
Average age (Yr, n = 87)	60.4 (34-89)
Location (n = 87)	Appendix: 2
	Cecum: 9
	Ascending colon: 17
	Transverse colon: 9
	Descending colon: 2
	Sigmoid colon: 1
	Rectum: 47
Depth (n = 66)	m-sm: 5
	Mp: 10
	a1/ss: 20
	a2/se: 23
	ai/si: 8
Lym (n = 44)	Positive 38
	Negative 6
V (n = 44)	Positive 32
	Negative 12
N (n = 59)	Positive 46
	Negative 13
H (n = 56)	Positive 11
	Negative 45
Prognosis (death n = 56)	> 6 mo: 22
	> 1 year: 19
	⩾ 1 year: 15

V: Venous invasions; N: Lymph node metastases; H: Liver metastases.

neoplasms easily invading and expanding, which are reported to occur frequently with metastasis and carry a poor prognosis. It was reported that the liver metastasis rate of 52.1% was higher compared to general colon and rectal carcinoma which had a rate of 10%[4]. Actual 1-year survival rates were 39%[5]. Therefore, one would predict the presence of vascular invasion to be associated with an increased incidence of liver metastasis and cause poor prognosis.

The phenomenon of tumor embolism of colon and rectal carcinoma is rare. As regards our research on tumor embolism of colon and rectum cancers in the mesenteric vein, we could find only 14 case reports including our case in Japanese papers (Table 2). In these, it was considered that histologically immature carcinomas were more likely to be complicated by a tumor embolism; but, there have been no reports of endocrine cell carcinoma showing mesenteric vein tumor embolism.

Little is known about the causes of tumor embolism of colon and rectum carcinomas; but, we consider that they involve vessel invasion. The biological and pathological processes of tumor emboli formation consis of several phases: (1) tumor growth in the primary lesion; (2) invasion of the primary tumor into the surrounding vessels; (3) detachment of tumor cells from the primary lesion; (4) dissemination of tumor cells by blood flow; (5) adhesion of tumor cells to endothelial cells or tumor embolism formation. In these processes, the metastatic potential of tumor cells depends in part on the ability to undergo cell aggregation, leading to the embolization of tumor cells in the microcapillaries, and

Table 2 A summary of 14 colon and rectal cases complicated by tumor emboli in mesenteric veins

Age	Sex	Location	Histology	Depth	Size (mm)	H	Recurrent	Prognosis
50	F	A/SMV	Poor	-	10 × 50	-	Liver, lung	4 mo
53	M	S/IMV	Mod	-	-	+	Liver, local	-
77	F	T/SMV	Poor	-	15 × 40	-	-	-
62	M	A/SMV	Poor	se	8	-	None	-
65	F	A/SMV	Mod	si	-	-	None	7 mo alive
68	M	S/IMV	Mod	a2	5 × 50	-	None	24 mo alive
75	M	A/SMV	-	-	40	-	None	-
82	F	A/SMV	Mod	-	-	-	-	-
67	M	A/SMV	Mod	se	-	+	Liver	-
56	F	A/SMV	Muc	si	-	-	-	-
78	F	R/IMV	Well	se	10 × 185	-	None	-
47	F	T/SMV	Mod	-	-	-	-	5 mo alive
78	F	A/SMV	Poor	ss	-	-	-	5 mo
79	F	S/IMV	End	se	-	-	Liver	9 mo alive

A: Ascending colon; T: Transverse colon; S: Sigmoid colon; R: Rectum; H: Liver metastases; SMV: Superior mesenteric vein; IMV: Inferior mesenteric vein. Poor: Poorly differentiated; Mod: Moderately differentiated; Well: Well differentiated; Muc: Mucinous; End: Endothelial.

additionally in macro-vessels. A strong correlation has been demonstrated between *in vitro* aggregation and *in vivo* metastatic potential[6]. Poorly differentiated cell carcinomas including endocrine cell carcinomas which are well known to have strong aggregation potential related to adhesion factors, for example integrin or CD56, are thought to form a tumor embolism easily.

Because of this invasion propensity, endocrine cell carcinomas develop distant metastasis, particularly in the liver. It is not clearly known whether such metastasis can be prevented or not; but, it has been suggested that early ligation of the tumor invading the vein in the operation is useful.

The treatment strategy for patients with endocrine cell carcinomas is to detect the origin and metastases at an early stage, and to monitor carefully after the operation because of its malignant potential. Surgical resection remains the mainstay of treatment, with modest impact on survival. In cases of metastasis or cases of adjuvant therapy, the literature suggests that treatment be initiated using CDDP + CPT11 based on lung small cell carcinoma[7,8]. A response rate of 41.5%[9] and median survival range of 10.4 mo have been reported[10]. In another report, four of 11 patients with endocrine cell carcinomas who were treated with S-1 survived for over 2 years after surgery[11,12].

In conclusion, this case highlights the aggressiveness of endocrine cell carcinomas of the colon and rectum. We showed that endocrine cell carcinomas are clinically more aggressive than colorectal adenocarcinomas, and they are capable of rapid distant spread; so, the prognosis is generally worse. So far, many studies have emphasized prognostic features, immunohistochemical characteristics, and pitfalls in diagnosis and treatment of endocrine cell carcinoma[13]. Practically, there are uncharted territories left. Further improvement in diagnosis and treatment is awaited.

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Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

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

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Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

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October 30-November 3, 2009
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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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

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

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INTRODUCTION

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EDITORIAL

Diet and epigenetics in colon cancer

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Abstract

Over the past few years, evidence has accumulated indicating that apart from genetic alterations, epigenetic alterations, through e.g. aberrant promoter methylation, play a major role in the initiation and progression of colorectal cancer (CRC). Even in the hereditary colon cancer syndromes, in which the susceptibility is inherited dominantly, cancer develops only as the result of the progressive accumulation of genetic and epigenetic alterations. Diet can both prevent and induce colon carcinogenesis, for instance, through epigenetic changes, which regulate the homeostasis of the intestinal mucosa. Food-derived compounds are constantly present in the intestine and may shift cellular balance toward harmful outcomes, such as increased susceptibility to mutations. There is strong evidence that a major component of cancer risk may involve epigenetic changes in normal cells that increase the probability of cancer after genetic mutation. The recognition of epigenetic changes as a driving force in colorectal neoplasia would open new areas of research in disease epidemiology, risk assessment, and treatment, especially in mutation carriers who already have an inherited predisposition to cancer.

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Key words: Colon cancer; Diet; DNA methylation; Epigenetics; Nutrition

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INTRODUCTION

The incidence of colorectal cancer (CRC) varies up to 25-fold between countries. Highest rates are found in Westernized societies, such as the USA, Australia and New Zealand and lowest rates are found in Africa and India. Evidence that causes of CRC are largely environmental comes from studies where people who migrate from low- to high-risk areas of the world reach the incidence of cancer in a high-risk country even over one or two generations. In these migration studies the main characteristic has been a change from a prudent diet to a Westernized diet with higher intake of energy dense foods and lowered physical activity.

It has been speculated that epigenetic changes in the genome might explain these ecological findings. Epigenetics are related to the inheritance of information based on gene expression levels, as opposed to genetics, which refers to information transmitted on the basis of gene sequence. In recent years, evidence has accumulated indicating that apart from genetic changes, epigenetic alterations play a major role in the initiation and progression of CRC[1,2].

Different environmental conditions may confer different activity to the same genes. Epigenetic processes are essential in normal development and differentiation but may sometimes be misdirected and predispose to cancer. Epigenetic events, such as altered methylation patterns (hypermethylation and hypomethylation), post-translational modifications of histones, and chromatin remodeling, can lead to inactivation of tumor suppressor genes, activation of oncogenes, or altered imprinting patterns. The best-known epigenetic marker is DNA methylation, described to occur in complex chromatin networks and is influenced by the modifications in histone structure that are commonly disrupted in cancer cells[3,4]. Diet is a major aspect of the environment which may influence DNA methylation thus providing an important common link between cancer and nutrition[5].

COLORECTAL CANCER

CRC is the second most common cause of cancer-related deaths in the Western world although a worlwide population-based study has shown that 5-year relative survival for CRC seems to be generally higher in high-income countries[6]. Approximately 50% of the population in Western countries will develop adenomatous lesions of the colon, but only a minor proportion will develop cancer[7]. CRCs are mainly sporadic, and inherited factors have been estimated to be of importance in about 30% of all CRCs[8]. While many inherited predisposing factors are still unidentified, 13% of CRCs have been reported to occur in association with the two most common inherited colon cancer-predisposition syndromes, i.e. hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP), which are caused by germline mutations in DNA mismatch repair (MMR) genes and the adenomatous polyposis coli (*APC*) tumor suppressor gene, respectively[7,9]. Susceptibility to HNPCC and FAP is inherited in an autosomal dominant manner. At the cellular level, these genes act recessively, i.e. inactivation of the wild-type allele (loss-of-function) is required for an altered cell phenotype[10]. The lifetime risk of cancer for individuals carrying an inherited germline mutation in a *MMR* gene or *APC* is high, but cancer develops only as the result of the progressive accumulation of somatic genetic and epigenetic alterations in several other genes involved in various cellular pathways.

MAJOR PATHWAYS OF COLORECTAL CARCINOGENESIS

Analyses of tumors associated with FAP and HNPCC have helped to understand many details of the molecular pathogenesis of CRC in general[11]. The development of CRC is a multi-step process beginning with the transformation of normal colonic epithelium, first to benign adenomatous polyps and eventually to invasive carcinoma, and finally metastasis[7,12]. Mutational inactivation of *APC* plays a rate-limiting role in about 70% of sporadic CRCs[13]. Epigenetic silencing of *APC* through promoter hypermethylation has also been reported in a number of sporadic colorectal adenomas and carcinomas[14]. The principal tumor promoting character of inactivated *APC* is the insufficient degradation of β-catenin, a key mediator of the Wnt signaling pathway. Consequently, more β-catenin enters the nucleus and overactivates Wnt signaling, resulting in transcriptional activation of Wnt/TCF4 (T-cell factor 4) target genes (e.g. *c-myc* and *cyclin D1*), initiating transformation of intestinal epithelial cells[15,16]. Physiologically, the Wnt pathway is essential for the maintenance of intestinal crypt progenitor compartments[17]. Tumors associated with *APC* mutations are characterized by chromosomal instability (CIN)[11].

Another pathway of carcinogenesis involves the cellular DNA MMR system. Cells defective in MMR are characterized by microsatellite instability (MSI) phenotype. MMR deficiency results in activation of the mutator pathway which creates accumulating frameshift mutations in many growth-regulatory genes with coding microsatellites, thus promoting genome-wide genetic instability[18]. Many of these affected genes are general tumor suppressor genes, as well as genes that function in DNA mismatch repair, Wnt signaling, and apoptotic pathways. MMR deficiency thus promotes the activation of many pathways, which lead to the expression of genes that favor cell growth[11]. Compared to CIN, MSI is a feature of a smaller subset of cancers; it is a hallmark for HNPCC tumors and is reported in approximately 15%-25% of all CRCs and 10%-20% of all endometrial and gastric cancers[7,19]. In HNPCC, most tumors are due to germline mutations in the MMR genes *MLH1*, *MSH2,* and *MSH6* (http://www.insight-group.org/). However, most sporadic MSI tumors are associated with epigenetic silencing (hypermethylation) of the *MLH1* promoter[20,21].

EPIGENETICS IN COLON CANCER

Epigenetics is defined as heritable changes in gene expression that are not due to any alteration in the DNA sequence[22]. It has been proposed that heritable changes in gene activity due to DNA modification should be referred to as epimutations to distinguish them from classical gene mutations. As DNA methylation is known to be essential for the normal control of gene activity during development, defects in methylation may have severe phenotypic consequences. Recently, considerable attention has been focused on the role of CpG islands (CGI) hypermethylation in the molecular pathogenesis of CRC. These islands are usually not methylated in normal cells[23,24]. The finding of aberrant *MLH1* promoter hypermethylation in sporadic MSI CRCs dramatically illustrated the role of epigenetic changes as potential pathogenetic alterations in cancer. Furthermore, in cell lines, reversion of the methylation using demethylating agents frequently restores expression of *MLH1,* demonstrating that methylation in fact induces gene silencing. These data strongly suggested that such aberrant *MLH1* promoter methylation is a cause of colon carcinogenesis[20]. The role of aberrant CGI hypermethylation in colon carcinogenesis was later demonstrated in animal studies, where overexpression of de novo DNA methyltransferase DNMT3b accelerated tumor formation[25], whereas chronic administration of an oral inhibitor of DNA methylation dramatically reduced tumor formation in the mucosa[26].

To date, several hypermethylated genes are associated with colorectal neoplasia, including tumor suppressor, DNA repair, and cell cycle regulatory genes (e.g. *APC, CDH13, CHFR, MLH1, BRCA1, p14, p16, RARB2, SFRP1, WRN, RASSF1A, MGMT,* and *TIMP3*)[2,27]. These genes are being explored as biomarkers in clinical use for preventive and therapeutic interventions. Of

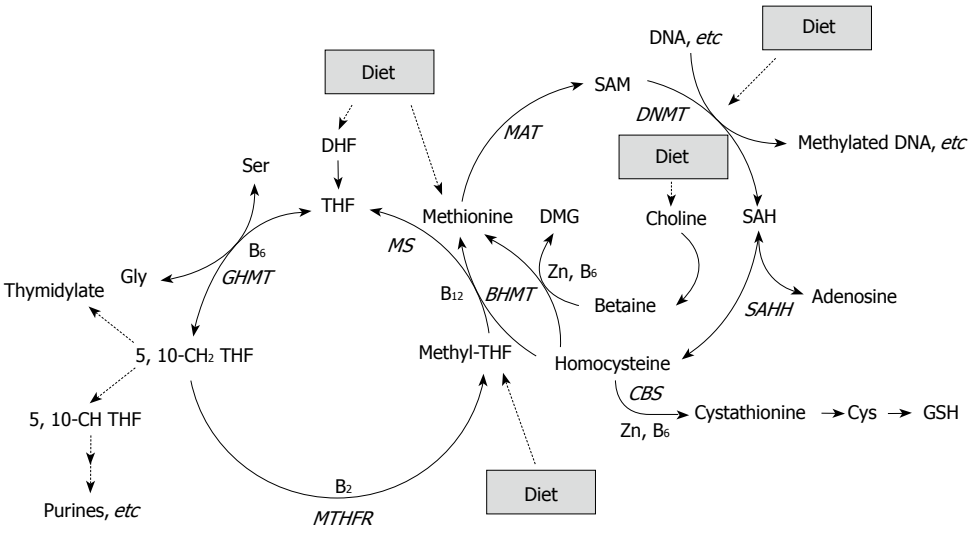

Figure 1 Folate, in the form of methyltetrahydrofolate (methyl-THF), is involved in remethylation of homocysteine to methionine, which is a precursor of SAM, the primary methyl group donor for most biological methylation reactions, also in DNA[64]. Folate deficiency may thus enhance CRC through an induction of genomic DNA hypomethylation. Expression of several enzymes (GHMT, MTHFR, BHMT, MAT, SAHH, CBS) involved in methyl metabolism can be regulated by diet such as availability of nutrients including essential amino acids, vitamins B2, B6 and B12, and Zinc (Zn).

these, promoter hypermethylation of *p16*^{INK4A}, *MGMT*, and *MLH1* have been suggested to be useful markers for risk assessment and hypermethylation of *APC* in the detection of colorectal carcinoma[27,28]. Hypermethylation of *MLH1* may also serve as a second "hit" inactivating the wild type copy of the gene in HNPCC-associated tumorigenesis[29].

Hypermethylation occurs at different cancer stages and can be associated with either of the two major pathways of colorectal carcinogenesis. For a panel of genes, the expression profiles measured in histologically normal mucosa have been reported to differ significantly between patients with and without colorectal cancers[30]. Moreover, different epigenetic phenotypes have been found to distinguish the colonic mucosa in individuals who develop sporadic MSI-positive and MSI-negative colorectal tumors[31]. These methylation phenotypes may underlie different developmental pathways that occur in these tumors. Recently, inactivation of tumor suppressor genes by promoter methylation was further shown to follow patterns characteristic of tumor type (CRC versus endometrial carcinomas) and family category (familial CRC *versus* sporadic) and was strongly influenced by *MLH1* promoter methylation status in all categories[32]. A phenomenon called CpG island methylator phenotype (CIMP) has been described in a subgroup of colorectal adenomas and carcinomas[33,34]. In CIMP tumors, multiple tumor suppressor genes are inactivated by promoter hypermethylation[35], and CIMP has been suggested to provide an alternative pathway to promote colon cancer resembling in many features MSI tumors, although they are microsatellite stable[36,37].

DIET AND COLON CANCER

Colorectal cancer is a disease associated with increasing age and there is strong evidence that the risk of CRC can be modified by lifestyle and environmental factors[38,39]. It has been demonstrated that diet may account for or prevent as much as 80% of CRC incidence[40]. Diet may affect gut mucosa either directly from the luminal side or indirectly through whole-body metabolism. Food-derived compounds that are constantly present in the intestine, or the blood content of nutrients, hormones and growth factors, may shift cellular balance toward harmful outcomes, such as increased susceptibility for genetic and epigenetic changes in a genome.

There is a strong assumption that diet, especially Western-type diet, contributes to the development of CRC. In 2007, the World Cancer Research Fund and the American Institute for Cancer Research published their 2nd comprehensive review entitled 'Food, Nutrition, Physical Activity and the Prevention of Cancer; a Global Perspective' (http://www.dietandcancerreport.org) supporting this belief. Based on mainly prospective cohort studies it was concluded that there is convincing evidence that red and processed meat, substantial consumption of alcoholic drinks, body fat and abdominal fatness, and the factors that lead to greater adult attained height or its consequences are causes of CRC. In addition, foods containing dietary fiber, garlic, milk and calcium probably protect against this cancer. Moreover, non-starchy vegetables, fruits, fish, foods containing folate, vitamin D, or selenium may protect against CRC, and foods containing animal fats or sugar may cause CRC. In a recent study, CRC re-occurrence was also shown to be significantly higher in subjects consuming the most Westernized diet compared to diets with more fiber and less fat and sugar[41].

The complex interactions of dietary components with each other and with metabolism make it difficult for epidemiological methods to specifically identify the components which might induce or prevent CRC (Figure 1). Murine models such as *Min*/+ mice, which

is the best-characterized mouse colonic neoplasia model and analogous to the human FAP syndrome[42] have provided a valuable tool, allowing thorough dissection of the effects of specifically controlled diets. A comprehensive list of compounds that have been tested for CRC promotion or prevention in this animal model can be found on Corpets website at http://www.inra.fr/reseau-nacre/sci-memb/corpet/Data/table.php?file=Min-mice.txt. Min (multiple intestinal neoplasia) is an autosomal dominant trait involving a nonsense mutation in codon 850 of the murine *APC* gene. As in humans, the mutation predisposes to intestinal tumorigenesis. In both *Min*/+ mice and healthy rats, different types of diet have been shown to cause considerable changes in intestinal cell signalling pathways (PKC, NF-κB, β-catenin and COX-2, cyclin D1, E-cadherin, and p53) both in tumor tissue and also in the surrounding mucosa[43-46]. In particular, red meat[47] and a Western-type diet with low levels of calcium and cholecalciferol and high levels of n-6 polyunsaturated fatty acids[48,49] were shown to have unfavorable effects on tumor formation. These results are in line with the epidemiological evidence on the effect of red meat on CRC. Inadequate dietary folate has been shown to impair DNA excision repair in the rat colon in the absence of any chemical carcinogen, and increased folate supplementation inhibited intestinal polyp formation in *Min*/+ mice[50,51]. Moreover, wild-type mice have been shown to develop colon adenomas and an early invasive carcinoma in long-term diet experiments with a Western-type diet containing reduced calcium, vitamin D, folic acid and increased fat content, but without carcinogen exposure[52].

Experimental work has indeed shown that due to the heterozygous nature of fiber or fiber-rich foods, it is very difficult to draw firm conclusions on the effects of fiber on CRC. It is evident that different fiber types (soluble *vs* insoluble) and sources (grain, vegetables and fruits) may act in totally opposite ways in CRC[53]. In addition, fibers and fatty acids in the diet interact with each other and affect outcome[54]. The same is probably true with fiber and red meat, since odds ratios for CRC were substantially reduced in subjects who had a high level of red meat in their diet but who also consumed high levels of fiber when compared to subjects with low fiber intake[55]. Phenolic compounds from fruits and berries[56,57], curcumin from tumeric[58,59], epicallocatechin from green tea[60], and n-3 fatty acids from fish[61] have been widely studied as possible chemopreventive agents and have been shown to regulate different cell signaling pathways[62]. Moreover, the effect of polyphenols on DNA methylation is under active investigation[63-65].

DIET AND EPIGENETICS

The elucidation of the effects of diets on epigenetic changes in the intestinal mucosa is of great importance, as aberrantly methylated genes may have the potential to be early-detection and prognostic markers for colon cancer. Unlike genetic changes in cancer, epigenetic changes, such as alterations in methylation, are potentially reversible and, therefore, provide promising targets for preventive and therapeutic interventions. Diet is a major aspect of the environment that may influence DNA methylation, and studies on the role of specific foods, diet-derived compounds and different types of dietary patterns on cellular mechanisms and epigenetics in CRC are increasing. Especially interesting are nutrients, which are needed for nucleic acid and DNA synthesis and for the enzymes regulating their syntheses, e.g. essential amino acids, zinc, folate, and vitamins B-6 and B-12[66] (Figure 1). The most studied nutrient in this area is folate, and the portfolio of evidence from animal, human, and *in vitro* studies suggest that the effects of folate deficiency and supplementation on DNA methylation are gene- and site-specific, and appear to depend on cell type, target organ, stage of transformation, and the degree and duration of folate depletion[67].

As in the classical experiment of agouti mice, in which maternal diet, high in folates, choline and vitamin B-12 shifted the coat color of the offspring[68,69], diet may also induce epimutations detectable in the phenotype later in life in humans. Monozygotic twins have been shown to be epigenetically indistinguishable during the early years of life, while older monozygotic twins exhibited remarkable differences in their overall content and genomic distribution of 5-methylcytosine DNA and histone acetylation[70]. Using the obesity-discordant monozygotic twins, Pietiläinen *et al*[71] have shown several changes in the transcription profiles of adipose tissue between the twins. The results showed the effects of acquired human obesity, which is independent of genetic factors, but may be related to epigenetic modulation of the genome.

A new and fascinating area in "diet and cancer" studies is the so called "fetal programming". In 1989, Barker *et al*[72] reported on an inverse relationship between birth weight and later glucose intolerance, hypertension, and hyperlipidemia and finally ischemic heart disease mortality in men born in England in the early 1900's. The hypothesis behind this relationship was that genes were epigenetically programmed in a way which favored energy storage in an energy poor environment and thus, later in an 'obesinogenic' or 'Western-type' environment, these same genes would lead to chronic diseases[73]. Genetic and early life environmental factors, even before birth, have also been shown to be important in adult height determination. Moreover, it has been suggested that the 'fetal programming' hypothesis or factors that promote linear growth in childhood might explain the epidemiological evidence on the clear dose-response relationship between greater adult height and a risk for CRC (http://www.dietandcancerreport.org). As has indeed been indicated by an animal model[74,75], the underlying mechanisms might include epigenetic modulation of growth hormone, insulin-like growth factors and sex hormone binding protein expression, all of which have an impact on height and growth and can be modulated by dietary means.

CONCLUSION

Epigenetic changes, such as alterations in methylation, may occur in normal cells, but may prime the mucosa for cancer progression. However, unlike genetic changes in cancer, these epigenetic changes are potentially reversible. The elucidation of the effects of food-derived compounds on epigenetic changes in intestinal mucosa is thus of great importance and will provide promising targets for preventive and therapeutic interventions. The identification of "methylation biomarkers" that are specific for colorectal tumorigenesis would be useful for risk assessment, especially in individuals who have an inherited susceptibility for CRC. Furthermore, those biomarkers required for the malignant phenotype would identify pathways important as therapeutic targets. In summary, the recognition of epigenetic changes as a driving force in colorectal neoplasia opens new areas of research in disease epidemiology, risk assessment, prevention, and treatment.

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GUIDELINES BASIC SCIENCE

Genetic mechanisms underlying the pathogenesis of tropical calcific pancreatitis

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Abstract

Chronic pancreatitis is known to be a heterogeneous disease with varied etiologies. Tropical calcific pancreatitis (TCP) is a severe form of chronic pancreatitis unique to developing countries. With growing evidence of genetic factors contributing to the pathogenesis of TCP, this review is aimed at compiling the available information in this field. We also propose a two hit model to explain the sequence of events in the pathogenesis of TCP.

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Key words: Chronic pancreatitis; Tropical calcific pancreatitis; Fibrocalculous pancreatic diabetes; Complex disease; Candidate gene analysis

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INTRODUCTION

Pancreatitis is a heterogeneous disease with varied etiologies, defined as an inflammatory disease of the pancreas leading to morphologic changes that typically cause pain and/or loss of function. Chronic pancreatitis (CP), [Online Mendelian inheritance in man (OMIM) 167800], is a continuing inflammatory disease which eventually leads to morphologic changes characterized by irreversible destruction and fibrosis of the exocrine parenchyma, leading to exocrine pancreatic insufficiency and progressive endocrine failure leading to diabetes. Histologic changes from the normal pancreatic architecture include irregular fibrosis, acinar cell loss, islet cell loss and inflammatory cell infiltrates, and distorted and blocked ducts[1]. Thus expert "state-of-the-science" reviewers conceded that "chronic pancreatitis remains an enigmatic process of uncertain pathogenesis, unpredictable clinical course, and unclear treatment"[2]. In most developed countries, alcohol causes about 60%-70% of the cases of chronic pancreatitis in male patients, and unknown causes are responsible for 25% of cases, termed as idiopathic chronic pancreatitis (ICP). Tropical calcific pancreatitis (TCP, OMIM 608189) is a juvenile form of chronic calcific non alcoholic pancreatitis, seen almost exclusively in developing countries of the tropical world[3]. In the most simple of terms, tropical calcific pancreatitis has been described as a disease with "pain in childhood, diabetes in puberty and death at the prime of life"[4].

TCP patients in former years were mostly children, adolescents, or sometimes young adults, who had common characteristics of malnutrition, deficiency signs, a cyanotic hue of enlarged lips, bilaterally enlarged parotid glands, a pot belly, and sometimes pedal edema. However, the clinical features and presentation of tropical pancreatitis has changed over the past 50 years with an older age of onset; severe malnutrition being uncommon with many patients being of ideal body weight which is attributed to improved nutritional status[5-8].

The cardinal manifestations of TCP are recurrent abdominal pain in childhood, followed by onset of diabetes mellitus a few years later. Prevalence of pancreatic calculi in TCP is nearly 90%, which is much higher than in alcoholic pancreatitis (30%)[9]. Pancreatic calculi varying in size and shape are demonstrable

throughout the markedly dilated main duct forming a ductogram and in some cases even in the dilated ductules mimicking a pancreatogram[9,10]. Early reports on TCP identified patients only in the late stages of the disease when extreme emaciation and other obvious clinical signs of protein malnutrition, such as bilateral parotid gland enlargement as well as skin and hair changes of kwashiorkor, dominated the clinical picture[11]. A recent population based study in southern India has shown the prevalence of TCP to be 0.02% in the general population[12]. Histopathological changes include dilation of the main pancreatic duct, intralobular fibrosis in early and interacinar fibrosis in later stages[13]. Unlike other forms of CP, the diabetes secondary to TCP has been given the unique name of 'fibrocalculous pancreatic diabetes' (FCPD).

ETIOPATHOGENESIS OF TCP

Etiopathogenic mechanisms of TCP are still unclear. Based on the observation that TCP almost exclusively affects the poor population of developing nations, malnutrition was strongly suspected to be a major etiologic factor. The role of under-nutrition in the etiology of TCP has been extensively reviewed[14-17]. However, recent observations suggest that malnutrition could be the effect rather than the cause of the disease. The geographical distribution of TCP coincides with the areas of consumption of cassava (Tapioca, *Manihot esculenta*), which is the staple diet of poor people in Kerala, a state in India. Cyanogen toxicity in the presence of malnutrition and antioxidant deficiency has been proposed as an ideal setting for free radical injury[18]. However, TCP is prevalent in many parts of India and Africa where cassava is not consumed and is not seen in West African populations consuming a high cassava diet[19]. A study on rats fed with a cassava diet for one year did not produce either pancreatitis or diabetes[20]. Thus it is unlikely that cassava ingestion can explain the majority of cases of TCP seen world wide and the current opinion is that cyanogen toxicity is not relevant in its etiopathogenesis. The contribution of dietary factors like proteins, and carbohydrates is not clear. The micronutrient deficiency-induced free radical hypothesis[21,22] remains to be proven and certainly merits further studies.

GENETICS OF TCP

It had been hypothesized about a century ago that the first important step in the development of pancreatitis is the inappropriate activation of trypsinogen in the pancreas[23,24]. Three different trypsinogens; cationic, anionic and meso, representing 23.1%, 16% and 0.5% of total pancreatic secretory proteins respectively, have been described in human pancreatic juice[25]. Normally, after trypsinogen is secreted into the duodenum it becomes active due to the action of an intestinal endopeptidase called enterokinase at the Lys15-Ile16 peptide bond, releasing the N-terminal octapeptide called trypsinogen activation peptide (TAP). It is thought that generally about 5% of trypsinogens get activated within the normal pancreas, but the pancreas has several safety mechanisms to cope with the premature activation of these enzymes, which would otherwise lead to indiscriminate proteolysis (autodigestion)[26].

Trypsin is known to lose its activity spontaneously by autolysis at the initial hydrolytic point of trypsin at Arg122-Lys123, which renders it more susceptible to further degradation[27]. A~6 kDa protein termed pancreatic secretory trypsin inhibitor (PSTI) or serine protease inhibitor Kazal type I (*SPINK1*, OMIM 167790) is present in the secretory granules of acinar cells which binds to the active site of trypsin in a 1:1 ratio and inhibits tryptic activities. Other safety mechanisms are the presence of trypsin inhibitors in plasma including α1-antitrypsin and β2-microglobulin, which inhibit the trypsin that leaks into the interstitial space around the pancreas[26]. It has been hypothesized that the primary mechanism to prevent trypsin injury inside the acinar cell is to maintain calcium at low levels[28]. Trypsinogen activation and trypsin survival are known to be regulated by calcium. Once trypsinogen is secreted into the duct, the calcium-dependent mechanisms utilized by the acinar cell for protection from trypsin become irrelevant because the calcium levels in the duct are quite high. Instead, the duct is protected through maintenance of an alkaline pH and by rapid flushing of the zymogens and prematurely activated enzymes out of the pancreas and into the duodenum[29]. Thus, trypsinogen plays a key role in the initiation of pancreatitis by evading the protective mechanisms leading to autodigestion of pancreas.

A high-density map of the human genome based on polymorphic simple tandem repeat (STR) markers and familial linkage analysis on several affected and unaffected individuals in several generations made it possible to identify an hereditary pancreatitis (HP) gene locus on chromosome 7q35[30,31]. Subsequently a mutation (365G>A) leading to arginine to histidine substitution at 122 position (R122H) in cationic trypsinogen gene [protease, serine, 1 (trypsin 1)(*PRSS1*), OMIM 276000], was found to be associated with hereditary pancreatitis[32]. Subsequent studies reported other *PRSS1* alterations including A16V, N29T, R116C, and R122C, as well as several others, in families with suspected hereditary pancreatitis or in patients without a family history (www.uni-leipzig.de/pancreasmutation)[33]. The current model of *PRSS1* mutations suggests that the identified mutations cause enhanced auto-activation of trypsinogen to trypsin or prevent prematurely activated trypsin from being inactivated by autolysis.

Familial aggregation is seen in about 8% of TCP patients. In some families, there has been evidence of vertical transmission of TCP from patients to offspring, while in others horizontal distribution of the disease among siblings was reported[34]. Familial aggregations suggest a genetic etiology for TCP. However, on screening known susceptibility factor, *PRSS1*, reported to be associated with HP and CP in Western populations, no association with TCP was found[35-37]. Instead, the

inhibitor of trypsinogen called *SPINK1* has been reported to be strongly associated with TCP[38,39]. An A>G transition at 101 nucleotide position in the *SPINK1* gene leading to substitution of asparagine by serine at codon 34 (N34S) has been reported with its highest frequency (approximate 46%) found so far in the Indian population[37]. Similar associations with varying strength have been reported by several studies, establishing *SPINK1* as a strong candidate for contributing to the pathogenesis of TCP[40,41]. Loss of function mutations in protease inhibitor *SPINK1* is thought to result in sustained "super-trypsin" activity. However, no genotype-phenotype correlation was found in patients carrying the N34S *SPINK1* mutation in homozygous or heterozygous states[42] and a wide variability has been reported in the pattern of inheritance[40]. Functional studies with human recombinant N34S *SPINK1* did not show altered trypsin inhibitor capacity or secretion[43-45]. An animal model deficient of Spink3, the murine orthologue of human *SPINK1*, showed progressive disappearance of acinar cells due to autophagic cell death and impaired regeneration. Thus, it might be surmised that *SPINK1* plays an essential role in the maintenance of integrity and regeneration of acinar cells[46]. Nevertheless, pathogenic mechanisms of N34S remain obscure. However, N34S has been observed to be in complete linkage disequilibrium with four intronic variants, 56-37T>C, 87+268A>g, 195-604G>A, 195-66_-65insTTTT[47], one of which may be pathogenic. Thus, in spite of being the strongest predictor and an important risk factor in the pathogenesis of TCP, the mechanism of N34S *SPINK1* still remains elusive.

Mutations in anionic trypsinogen [protease, serine, 2 (trypsin 2) (*PRSS2*), OMIM 601564] have been hypothesized to cause the disease by a mechanism similar to that of *PRSS1*. Earlier studies by various groups in ICP and TCP patients did not find associated polymorphisms in *PRSS2*[48,49]. However, a glycine to arginine change at codon 191 in *PRSS2* screened in a European population has been demonstrated to play a protective role against chronic pancreatitis[50]. Functional studies on purified recombinant G191R protein revealed that generation of a novel tryptic cleavage site within the mutated gene product makes the enzyme hypersensitive to autocatalytic proteolysis, thus playing a protective role in chronic pancreatitis. However, data from a study by Chandak's group (manuscript under review) suggests that this variant may not have a significant role to play in the Indian population. A very low allele frequency in the control populations and a comparable frequency in TCP patients are suggestive of the variant allele being neutral to natural selection. This could possibly be due to the dietary patterns marked by low protein consumption.

An association of Cystic fibrosis transmembrane regulator (*CFTR*, OMIM 602421) gene with alcoholic pancreatitis and ICP has been reported, where about 13.4%[51] and 25.9%[52] of patients in two studies were shown to carry at least one mutation in the gene. A study by Noone *et al*[53] revealed association of *CFTR* mutations with ICP and a possibility of its interaction with *PRSS1* and *SPINK1* mutations in western populations. However, the frequency of *CFTR* mutations was found to be lower in TCP patients[54], and needs to be studied in a larger group of patients.

Previous studies with synthetic substrates demonstrated a similarity between cathepsin B (*CTSB*, OMIM 116810) and trypsin in their specificity towards synthetic substrates. This made it of interest to observe if cathepsin B might activate trypsinogen. There is evidence to suggest that partially purified beef spleen cathepsin B activates trypsinogen to a trypsin-like product. Studies on native and recombinant cationic trypsinogen assigned a central role of cathepsin B in the development of different forms of pancreatitis[55]. It was recently shown that polymorphisms in *CTSB* are associated with TCP[56]. Mutations in the propeptide region of the *CTSB* gene like L26V and S53G have been found to be associated with TCP and it has been hypothesized that inappropriate localization of cathepsin B protein in zymogen granules due to these mutations could lead to premature activation of trypsinogen. This not only suggests an important role for *CTSB* polymorphisms in TCP, but also advocates emphasis on factors likely to change the pH or alter the intracellular calcium levels.

An important feature of TCP is the high incidence of pancreatic calcification and stone formation. It has been suggested that lithostathine C [coded by regenerating islet-derived protein (*Reg*) genes], a major proteic component of pancreatic stone in patients with alcoholic calcifying chronic pancreatitis, could promote the nucleation of calcite crystals or may prevent pancreatic lithiasis by inhibiting calcite crystal nucleation and growth in the pancreatic juice. With suggestions that it might help in preventing the harmful activation of protease precursors in the pancreatic juice, it was thought to be a logical assumption that mutations in this gene could lead to pancreatitis and calcification[57]. Exons of *Reg1*α gene (OMIM 167770) were screened for associated polymorphisms, but no association has been found so far[58,59]. As the protein is known to be down-regulated in TCP patients, a recent study screened the gene including the putative promoter and intronic regions, but did not find a significant association with TCP[60]. *Reg1*α is highly represented in human pancreatic secretions unlike *Reg1*β (OMIM 167771), which is 87% homologous to *Reg1*α and is not extensively studied and remains to be characterized[61].

Progression to diabetes, called fibro-calculous pancreatic diabetes (FCPD), which takes place in a majority of TCP patients, is another important feature of TCP, but the nature of the diabetes is controversial. A recent study, hypothesized that investigating a known susceptibility factor for T1D or T2D can help in understanding the type and mechanism of diabetes in FCPD patients. In this study type 2 diabetes (T2D) associated polymorphisms in transcription factor 7 like protein 2 (*TCF7L2,* OMIM 602228) were screened in TCP and FCPD patients. Although no association was found with FCPD independently, data suggested that the

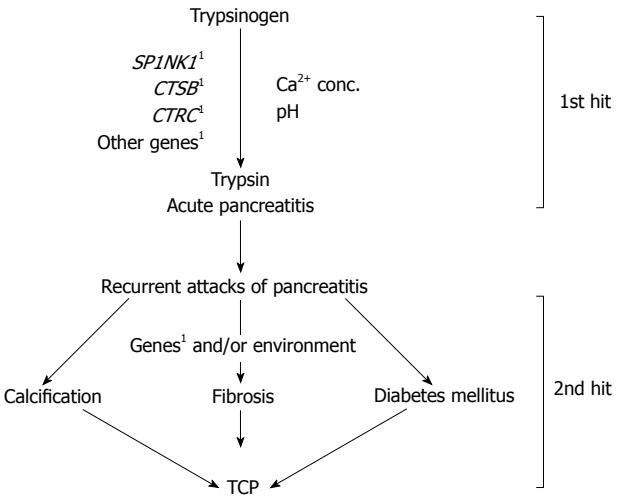

Figure 1 Two hit model for the pathogenesis of TCP. First hit contributing to the pathogenesis of TCP is likely to be loss of balance between activation events and degradation of active trypsin leading to presence of persistent "super-trypsin" within the acinar cell, which could occur due to mutations in one or more genes like *SPINK1, CTSB, CTRC*, other yet unidentified genes, resulting in inflammation. Presence of additional genetic and/or environmental factors, which constitute the second hit, may lead to one or more phenotypes such as stone formation, fibrosis, and/or diabetes mellitus. [1]Mutation in genes.

polymorphisms in *TCF7L2* may interact with *SPINK1* and *CTSB* mutations and cause FCPD[62].

Increased accumulation of extracellular matrix is a histological characteristic of chronic pancreatitis that results in pancreatic fibrosis (Haber *et al*, 1999). Angiotensin converting enzyme (ACE, OMIM 106180), a zinc metallopeptidase which is a key enzyme of the renin-angiotensin system (RAS) and is known to proliferate hepatic stellate cells, has been hypothesized to play a role in pancreatic fibrosis in TCP patients. A polymorphism within intron 16 (g.11417_11704del287) of the ACE gene is strongly related to the circulating enzyme levels in a dose dependent manner. However, no association of this polymorphism has been found with TCP[63].

Genetic and functional data from a recent study by Rosendahl *et al*[64] identified chymotrypsin C (*CTRC*, OMIM 601405) as a new pancreatitis-associated gene and discovered that loss-of-function alterations in the gene predispose to pancreatitis by diminishing its protective trypsin-degrading activity. The same was shown to be true with TCP patients. Their observations provided support for the trypsin-dependent pathogenic model of chronic pancreatitis in humans by demonstrating that trypsin-trypsinogen degradation by *CTRC* is an important mechanism for maintaining the physiological protease-antiprotease balance in the pancreas. Copy number variations, i.e. triplication of a 605 kilobase segment containing the *PRSS1* and *PRSS2* genes have been reported in hereditary pancreatitis patients[65]. A study by Masson *et al*[66] revealed the molecular basis of 6% of young ICP patients demonstrating chronic pancreatitis to be a genomic disorder. However, no copy number variations were found in TCP patients to provide evidence, showing that trypsinogen gene mutations do not play an important role in the pathogenesis of TCP in the Indian population.

Mutations involving the calcium sensing receptor (*CASR*, OMIM 601199) have been suggested to increase the risk of chronic pancreatitis (CP), since high intracellular levels of calcium activate trypsinogen within the acinar cells. A combination of *CASR* and *SPINK1* gene mutations has been proposed to predispose to idiopathic CP[67]. A study by Murugaian *et al*[68] identified 4 novel *CASR* mutations in TCP patients and concluded that the risk of disease may be further increased if there is an associated *SPINK1* mutation.

CONCLUSION

In conclusion, all the established mutations in the cationic trypsinogen gene, including the copy number polymorphism, are not a common cause of tropical calcific pancreatitis in the Indian population[37,66]. The model for etiopathogenesis of TCP emerging from the available information is presented in Figure 1. Many aspects of TCP remain unclear. What triggers intra-pancreatic trypsin activation, and in the presence of an intact autolysis site how is it maintained in an active state? Are the various manifestations of TCP, such as calcification, ketosis resistant diabetes mellitus, pancreatic cancer and fibrosis, consequences of a proteolytic cascade of prematurely activated trypsin? Since TCP is a complex disease, in addition to candidate gene analysis which has undoubtedly been influential, there is a necessity for a more comprehensive and holistic approach to understand its etiopathogenesis, to help early detection and discover possible treatment. The role of environmental factors as disease modifiers cannot be undermined. An in-depth study of the contribution of dietary- and lifestyle-related factors, and their association with genetic variants would yield interesting leads.

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REVIEW

Breastfeeding and genetic factors in the etiology of inflammatory bowel disease in children

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Abstract

Inflammatory bowel disease is a chronic, debilitating disorder of the gastrointestinal tract. The etiology of inflammatory bowel disease has not been elucidated, but is thought to be multifactorial with both environmental and genetic influences. A large body of research has been conducted to elucidate the etiology of inflammatory bowel disease. This article reviews this literature, emphasizing the studies of breastfeeding and the studies of genetic factors, particularly NOD2 polymorphisms.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Etiology; Risk factors; Protective factors; NOD2/CARD15; Single nucleotide polymorphisms

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INTRODUCTION

Inflammatory bowel disease (IBD) is an idiopathic condition characterized by chronic destructive inflammation of the gastrointestinal tract. The morbidity of IBD, particularly in younger patients, can be considerable and may include effects on growth and development, reproductive health, education, employment, and psychological health. The pathogenesis of IBD is thought to be a complex interaction between genetic predisposition and inappropriate activation of the mucosal immune system driven by the presence of enteric flora and resulting in tissue injury[1-3]. Genetic factors have been the subject of intense investigation and, at least in some cases, may be involved in inappropriate activation of the mucosal immune system. Discerning other factors that influence the activation of the mucosal immune system or the distribution of enteric flora present in those at risk for IBD is paramount in lessening the impact of IBD, a debilitating condition that affects children and adults throughout the world. One factor that may be important in the pathogenesis of IBD is breastfeeding. Breastfeeding is a protective factor for the development of several chronic disorders[4]. The intent of this article is to review factors involved in the development of IBD in children, with particular emphasis on genetic factors and breastfeeding.

BACKGROUND

IBD is generally considered to include two major disorders, Crohn's disease (CD) and ulcerative colitis (UC). CD and UC are similar conditions, but most experts consider them separate diseases[2,3]. This distinction might have important therapeutic implications. In the individual patient, CD and UC can usually be distinguished on the basis of clinical features (Table 1) and laboratory manifestations, as well as radiographic, endoscopic, and histological features.

Table 1 Clinical and epidemiological features of Crohn's disease and ulcerative colitis

	CD	UC
Region of involvement	Any portion of gastrointestinal tract	Rectum and colon
Typical region of involvement	Ileum and colon	Rectum and extending proximally
Nature of inflammatory process	Segmental, transmural	Continuous, limited to mucosa
Extraintestinal manifestations	Oral aphthous ulcers, peripheral arthritis, erythema nodosum, digital clubbing, episcleritis, renal stones, and gallstones	Pyoderma gangrenosum, sclerosing cholangitis, chronic active hepatitis, and ankylosing spondylitis
Age at presentation	Bimodal; 1st peak in late teens; 2nd peak in late adulthood	Bimodal; 1st peak in late teens; 2nd peak in late adulthood
Gender difference	Women are slightly more likely than men to develop CD	Men are slightly more likely than women to develop UC

In some cases, a definitive diagnosis cannot initially be made. These patients are diagnosed with indeterminate colitis until a definitive diagnosis can be determined[2,5].

The clinical presentation of CD depends on the region of the bowel involved, the degree of inflammation, and the presence of complications. Children with ileocolitis typically present with crampy abdominal pain and diarrhea, which may be bloody. Systemic signs and symptoms such as fever, malaise, easy fatigability, and growth failure, are common in CD. Gastric and duodenal involvement may cause vomiting and epigastric pain. Perianal disease is common in CD. The clinical presentation of UC typically includes bloody diarrhea with mucus. More severe cases may also present with tenesmus, urgency, crampy abdominal pain, and nocturnal bowel movements. Onset is usually insidious with gradual progression of symptoms. Fever, severe anemia, hypoalbuminemia, and leukocytosis may also be present. Presentation may be milder in cases involving only the rectum. Systemic manifestations occur less commonly than in CD. UC is associated with an increased risk of colon cancer. Secondary amenorrhea is common during periods of active disease in both CD and UC[5,6].

Both CD and UC were identified as clinical disorders in the early 20th century. Population-based studies have suggested an uneven distribution of IBD throughout the world with the highest disease rates occurring in "Westernized" countries[6]. The reported incidence of CD is 3-4/100 000 and the prevalence is 30-100/100 000. Earlier age at onset is associated with more severe disease and with increased likelihood of CD in family members. Incidence rates for UC are highest in European countries and the United States (15/100 000) and lowest in Japan and South Africa (1/100 000). The incidence in Israel varies by country of origin with the lowest rates among those from Asia or Africa. The prevalence of UC in European countries and the United States is 100-200/100 000. In Europe and North America, the incidence of IBD has increased steadily since the first half of the 20th century[7,8]. In particular, the incidence of CD has increased although the incidence of UC has generally reached a plateau in the second half of the 20th century (Tables 2 and 3). Of note, the incidence of both CD and UC in children has been increasing.

Rapid changes in the incidence of IBD within the same population can best be explained by changes in environmental factors since changes in genetic predisposition do not occur rapidly[7]. Some studies have demonstrated a predilection of IBD for urban rather than rural populations or a north-south gradient of disease incidence[9]. The incidence of IBD in individuals of Jewish ancestry is higher than in individuals not of Jewish ancestry. Migrant studies have shown that immigrants acquire IBD at a rate consistent with that of the new geographic area[7]. This evidence suggests that there is a significant influence of environmental factors on the development of IBD.

A number of factors, including smoking, oral contraceptive (OC) agents and diet, have been considered as potential risk factors for IBD. Smoking, the most extensively studied environmental factor in the development of IBD, is a risk factor for CD[10], but a protective factor for UC[11]. A meta-analysis of smoking and IBD confirmed these findings (Table 4)[12]. The effect of passive smoke exposure in childhood on the subsequent development of IBD remains inconclusive (Table 4). The relationship between OC agents and the development of IBD is less certain. Epidemiologic investigations of the relationship between OC agents and IBD yielded mixed results. A meta-analysis of these studies showed evidence of a modestly increased risk for CD and UC in OC users (Table 4)[13]. A more recent study confirmed the modestly increased risk of CD and UC in former and current users of OC agents, but the difference was statistically significant only for current users of OC agents and CD [ψ = 3.4 (1.0-11.9), n = 106 age-matched pairs][14]. Because of the direct interface between diet and the gastrointestinal tract, the role of dietary factors in the development of IBD has been extensively investigated. Early studies of diet had serious methodological flaws and their findings have been questioned[15]. A Japanese study of 101 cases and 143 controls found an increased risk of UC associated with consumption of Western foods (P_{trend} = 0.04)[16]. More recently, a well-designed study of diet was conducted in newly diagnosed IBD patients [CD (n = 33), UC (n = 54)] (Table 4)[17]. A decreased risk of CD was associated with increasing consumption of vitamin C. An increased risk of UC was associated with increasing consumption of sucrose, animal fat, cholesterol, and soft drinks.

The role of preceding infections in the development of IBD is unclear (Table 4). In a matched case-control study, patients with CD had a higher rate of gastroenteritis in the first six months of life than controls, but patients with UC did not[18]. In separate studies, children with CD[19] and children with UC[20] were more likely than their unaffected siblings to have had diarrheal illness during infancy. In a large study

Table 2 Incidence of Crohn's disease

Annual incidence per 100 000	Time period	Geographic region	Data collection	Age group studied	References
0.73	1958-1960	Nottingham, England	Retrospective	Adults	[70]
3.63	1970-1972				[70]
6.1	1971-1980	Orebro, Sweden	Retrospective	Children ≤ 16 years old	[71]
[1]3.39 (white males)	1977-1979	Baltimore SMSA	Retrospective	All	[10]
[1]3.54 (white females)					[10]
[1]1.29 (non-white males)					[10]
[1]4.08 (non-white females)					[10]
0.66	1968	Scotland	Retrospective	Children ≤ 16 years old	[72]
2.29[b]	1983				[72]
0.00	1920-1929	Rochester, NY	Retrospective	All	[73]
5.03	1970-1979				[73]
3.90	1980-1989				[73]
< 1.0	1962-1969	Copenhagen	Prospective	All	[74]
4.1	1979-1987				[74]
1.30	1983-1988	South Glamorgan, Wales	Retrospective	Children < 16 years old	[75]
3.11	1989-1993				[75]
[2]1.0	1940-1943	Olmsted County, MN	Retrospective	All	[76]
[2]7.8	1964-1973				[76]
[2]6.9	1984-1993				[76]
14.6	1989-1994	Manitoba, Canada	Retrospective	All	[77]
[2]1.91	1981-1983	Scotland	Retrospective	Children < 19 years old	[78]
[2]2.91	1990-1992				[78]
5.5	1990-1994	Iceland	Prospective	All	[79]
1.2	1984-1986	Sweden	Prospective	Children < 16 years old	[80]
1.3	1993-1995				[80]
[2]5.2	1988-1990	Northern France	Prospective	All	[81]
[2]5.8	1991-1993				[81]
[2]5.9	1994-1996				[81]
[2]6.4	1997-1999				[81]
2.00	1990-1994	Southeastern Norway	Prospective	Children < 16 years old	[82]
4.56	2000-2001	Wisconsin	Prospective	Children < 18 years old	[83]

[1]Age-adjusted; [2]Age- and gender-adjusted; [b]$P < 0.0001$ compared to 1968.

(257 cases of IBD with 2 matched controls per case) of perinatal risk factors for IBD, the greatest risk was associated with postnatal infections in the child [ψ = 5.5 (2.6-11.8)][21]. In a nested case-control study (26 CD, 29 UC, eight randomly selected controls matched for gender and social class) from two national longitudinal birth cohorts, infections during pregnancy or in childhood were associated with an increased risk of CD and UC, but these differences were not statistically significant[22]. In a large, international multicenter study of children with IBD and sex and age-matched controls, recurrent respiratory infections were significantly more common in CD and UC patients than their controls and patients with CD used antibiotics more frequently than their controls[23]. In this study, there were no differences between cases and controls in the frequency of gastroenteritis severe enough to require hospitalization, the age of its occurrence, the frequency of other hospitalizations, other recurrent infections, or tonsillectomy/adenoidectomy[23]. In another study, adults with CD reported an increased frequency of childhood infections compared to neighbor controls as well as more frequent treatment with antibiotics for both otitis and pharyngitis[24]. In this same study, adults with UC reported more frequent childhood infections than neighbor controls, but no increased frequency of antibiotic treatment[24].

The role of perinatal and childhood factors in the development of IBD has been investigated, but very little has been demonstrated (Table 4). No significant differences were found in birth weight, prematurity, birth at home, nursery school attendance, number of children in nursery or first class at school, number of playmates, bedroom sharing with other children, home environment at different ages, birth month, or the number of siblings but children with CD were more likely than controls to be last born ($P < 0.02$). The age interval to the previous sibling did not differ between last-born patients and controls[23]. Two smaller studies found no association between seasonality of birth, maternal age at birth, birth weight, or birth order and either disease[25,26]. In a study of perinatal factors, the occurrence of any perinatal health event increased the risk of both CD and UC[21]. The occurrence of any noninfectious perinatal event was an independent risk factor for IBD [ψ = 3.5 (2.0-6.3)] as was low socioeconomic status [ψ = 2.7 (1.2-5.7)] and low placental weight [ψ = 1.5 (1.0-2.2)]. A large, population-based case-control study from Sweden used siblings as a marker for exposure pattern[27]. Cases were identified through the Swedish Inpatient Register and controls (matched by age and area of residence) through Swedish Census, Birth and Death registers. Analyses were adjusted for sex, multiple birth, maternal age, region, year of birth, and fathers' social class. There was a significant, graded negative association between CD and number of younger siblings. Increasing maternal age was

Table 3 Incidence of ulcerative colitis

Annual incidence per 100 000	Time period	Geographic region	Data collection	Age group studied	References
[1]2.92 (white males)	1977-1979	Baltimore SMSA	Retrospective	All	[10]
[1]1.79 (white females)					[10]
[1]1.29 (non-white males)					[10]
[1]2.90 (non-white females)					[10]
1.91	1968	Scotland	Retrospective	Children ≤ 16 years old	[72]
[3]1.56	1983				[72]
0.06	1920-1929	Rochester, NY	Retrospective	All	[73]
3.51	1970-1979				[73]
2.32	1980-1989				[73]
6.9	1962-1969	Copenhagen	Prospective	All	[84]
9.2	1980-1987				[84]
0.71	1983-1993	South Glamorgan, Wales	Retrospective	Children < 16 years old	[75]
14.3	1989-1994	Manitoba, Canada	Retrospective	All	[77]
16.5	1990-1994	Iceland	Prospective	All	[79]
1.4	1984-1986	Sweden	Prospective	Children < 16 years old	[80]
3.2[a]	1993-1995				[80]
[2]4.2	1988-1990	Northern France	Prospective	All	[81]
[2]4.3	1991-1993				[81]
[2]3.9	1994-1996				[81]
[2]3.5	1997-1999				[81]
2.14	1990-1994	Southeastern Norway	Prospective	Children < 16 years old	[77]
2.14	2000-2001	Wisconsin	Prospective	Children < 18 years old	[83]

[1]Age-adjusted; [2]Age- and gender-adjusted; [3]$P = 0.052$ compared to 1968; [a]$P < 0.05$ compared to 1984-1986.

negatively associated with CD ($P < 0.001$). There was a protective effect of younger siblings that was greatest for those born soon after subjects, with statistical significance disappearing for those born 5 years later than subjects. There was a significant, graded positive association between UC and number of older siblings. Maternal age was not consistently associated with UC. There was no discernible pattern in the relationship with UC risk by age difference between subjects and their older siblings[27].

Several studies have shown an increased risk of IBD in relatives of individuals with CD compared to the general population. Population studies found that first-degree relatives of CD patients had a prevalence of CD that was 10-21 times the population prevalence and a prevalence of UC that was 6-10 times the population prevalence[28,29]. Relatives of a patient with CD had a greater risk of acquiring CD than UC and relatives of a patient with UC had a greater risk of acquiring UC than CD, but both diseases could occur in the same family[28,30]. In general, the familial association has been greater for individuals with CD than for those with UC[28]. Twin studies have demonstrated greater concordance for CD than for UC and greater concordance for monozygotic twins than for dizygotic twins[31-33]. These observations suggest that there is an inherited predisposition to the development of CD and UC.

GENETIC FACTORS

Several groups of investigators have conducted studies to discern the modes of inheritance of CD and UC. In one study, segregation analysis of 265 CD patients and 5387 relatives suggested a recessive susceptibility gene for CD with incomplete penetrance. The model predicted that the proportion of cases explained by the presence of this gene would be very high among those with early onset disease and about 30% of cases would be due to homozygosity for the gene[34]. In a second study, complex segregation analysis of 133 CD patients and their relatives also suggested a recessive major locus, however, with nearly complete penetrance. This model also predicted that 7% of patients would be homozygous for the recessive gene, but 28% of patients under age 20 would be homozygous for the recessive gene[35]. In UC patients, segregation analysis of 65 patients and their relatives suggested a rare additive major gene that would account for 11% of the total phenotypic variance and would have penetrance for heterozygotes of 0.22. The model predicted that affected individuals would be more likely to be heterozygous than homozygous for the additive gene and risk to an offspring of an affected individual would be 11%[36].

Subsequent genome-wide scanning studies have identified a series of IBD susceptibility loci[37-39]. Some of these loci are more strongly associated with CD, others with UC, and some with both. With respect to CD, the most recent genome-wide scanning study confirmed previously established associations at IBD1, IBD5 (5q31), IL23R, ATG16L1, IRGM, TNFSF15, and PTPN2, but also identified 21 new loci associated with CD[40]. The first locus identified, IBD1, has shown evidence for linkage with CD, but not with UC[41]. Definitive evidence for linkage at IBD1 was confirmed in a large, international IBD genetics consortium study that also demonstrated equally increased allele sharing at this locus in Jewish and non-Jewish cohorts[42]. In 2001, three major, relatively uncommon, single nucleotide polymorphisms (SNPs) were identified at a gene in

Table 4 Factors affecting development of IBD

Factor	Effect	Findings
Cigarette smoking	Protective factor for UC	Pooled OR for UC = 0.41 (0.34-0.48); χ^2 = 11.52 (P < 0.001)[12]
	Risk factor for CD	Pooled OR for CD = 2.0 (1.65-2.47); χ^2 = 48.4 (P < 0.001)[12]
Passive cigarette smoke	Uncertain	No effect[25,85]
		UC [ψ = 0.50 (0.25-1.00), *n* = 163][86]
		CD [ψ = 5.32 (1.09-25.9), *n* = 39 age and sex-matched pairs][87]
		UC [ψ = 2.19 (0.75-6.41), *n* = 33 age and sex-matched pairs][87]
Oral contraceptive use	Risk factor for UC	Pooled RR for UC = 1.29 [(0.94-1.77) adjusted for smoking]
	Risk factor for CD	Pooled RR for UC = 1.68 [(0.97-2.88) unadjusted for smoking]
		Pooled RR for CD = 1.44 [(1.12-1.86) adjusted for smoking]
		Pooled RR for CD = 1.68 [(0.97-2.88) unadjusted for smoking]
Diet	Protective factor for CD	
	Vitamin C	ψ = 0.48 and ψ = 0.23 for medium and high intake, respectively, *vs* low intake, P_{trend} = 0.02[17]
	Risk factors for UC	
	Sucrose	[Sucrose] ψ = 2.05 and ψ = 4.22 for medium and high intake, respectively, *vs* low intake, P_{trend} = 0.02
	Animal fat	[Animal fat] ψ = 2.02 and ψ = 4.09 for medium and high intake, respectively, *vs* low intake, P_{trend} = 0.02
	Cholesterol	[Cholesterol] ψ = 2.14 and ψ = 4.57 for medium and high intake, respectively, *vs* low intake, P_{trend} = 0.02
	Soft drinks	[Soft drinks] ψ = 1.84 and ψ = 3.39 for medium and high intake, respectively, *vs* low intake, *vs* P_{trend} = 0.02[17]
Infections	Risk factor for CD/possible risk factor for UC	CD patients had a higher rate of gastroenteritis than did controls (6/57 *vs* 1/114, *P* = 0.005)[8]
		UC patients and controls did not differ (4/51 *vs* 1/102, *P* = NS)[8]
	Gastroenteritis	Children with CD were more likely than unaffected siblings to have had diarrheal illness [RR = 2.7 (95% CI 1.5-5.8) *P* < 0.02, *n* = 294] Children with UC were more likely than unaffected siblings to have had diarrheal illness [RR = 3.2 (95% CI 1.15-8.75), *P* = 0.03, *n* = 231][20]
	Diarrheal illness in infancy	
	Risk factor for CD and UC	Recurrent respiratory infections were significantly more common in CD patients and in UC patients than their controls (102/298 *vs* 156/601 and 73/194 *vs* 106/393, respectively, both *P* < 0.01)[7]
	Recurrent respiratory infections	Adults with CD had an increased frequency of childhood infections compared to neighbor controls [ψ = 4.67, (95% CI 2.65-8.23) *n* = 322 cases, 262 controls][24]
	Childhood infections	Adults with UC had more frequent childhood infections than neighbor controls [ψ = 2.37 (95% CI 1.19-4.71) (*n* = 181 cases, 141 controls)][24]
Antibiotic use	Risk factor for CD	Patients with CD used antibiotics more frequently than controls (*P* < 0.01)[7]
		Adults with CD had more frequent treatment with antibiotics for both otitis [ψ = 2.07 (95% CI 1.03-4.14)] and pharyngitis [ψ = 2.14 (95% CI 1.20-3.84)] than controls[24]
Perinatal factors	Risk factor for UC	For UC, the odds ratios for having one, two, and three or more older siblings were 1.08 (1.03-1.14), 1.09 (1.01-1.16), and 1.12 (1.02-1.23), respectively (*n* = 15 823 cases; 79 546 controls)[27]
	Number of older siblings	
	Protective factor for CD	For CD, the odds ratios for having one, two, and three or more younger siblings were 0.93 (0.88-0.99), 0.89 (0.82-0.96), and 0.83 (0.75-0.92), respectively (*n* = 12 668 cases; 63 035 controls)[27]
	Number of younger siblings	

this locus, the *NOD2/CARD15* gene that conferred susceptibility to CD[41,43]. One group identified three separate SNPs in the *NOD2* gene [a frameshift variant (L1007fsinsC) and two missense variants (R702W and G908R)] which were associated with CD. The genotype relative risks for CD in their sample compared to those with no mutations, for simple heterozygous individuals, homozygous individuals, and compound heterozygous individuals (i.e. those with two different variant alleles) were 3, 38, and 44, respectively. The demonstrated gene-dosage effect suggested a recessive model of inheritance. The other group identified only the frameshift variant SNP. The reported genotype relative risks for heterozygous and homozygous individuals were 1.5 and 17.6, respectively. A third group confirmed the presence of the frameshift variant in two different cohorts of patients[44]. For CD, the mutation was highly associated [heterozygotes and homozygotes *vs* normal, ψ = 2.6 (1.5-4.5), ψ = 42.1 (4.3-∞), respectively]. In all of these studies, the gene mutation was not associated with UC. Subsequent studies by different investigators in different Caucasian cohorts have confirmed that these NOD2 variants were independent risk factors for CD, conferring susceptibility for CD[45-49]. Overall, 27%-32% of CD patients carry one major variant allele compared to 10%-20% of Caucasian controls and 8%-17% of CD patients carry two major variant alleles compared to 1%-5% of Caucasian controls[35,50].

The *NOD2* gene encodes for a protein in monocytes that is involved in the immune-mediated inflammatory response to enteric pathogens. The frameshift variant truncates the NOD2 protein and is associated with a marked hyporesponsiveness of NF-κB activation with lipopolysaccharide treatment. The missense variants yield a NOD2 protein that showed a greater response to lipopolysaccharide, but still a diminished ability to activate NF-κB. How the mutant NOD2 proteins and impaired NF-κB activation confer susceptibility to CD is unknown. However, it is known that NOD2 protein plays a critical role in the detection of bacterial muramyl dipeptide, and can activate the adaptive immune system by acting as an adjuvant receptor for antibody production[37,51].

A number of investigators have attempted to define demographic and clinical features associated with the NOD2 variants known to be associated with CD. Some have identified a younger age of onset of CD associated with the NOD2 variants, particularly the frameshift variant and particularly for homozygotes or compound heterozygotes[45,47] while others have not[46,49,52,53]. No studies have reported any relationship between gender and the NOD2 variants. In one study, the frequency of NOD2 variant alleles was significantly higher in familial

cases of CD than in sporadic cases of CD [30.9% (n = 173) *vs* 19.3% (n = 405), $P < 0.001$][46], but in two other studies the frequency of NOD2 variant alleles did not differ between familial and sporadic cases[47,49]. The reported NOD2 variants confer risk primarily in Caucasians, since they were not found in Asians with CD[54], and were found in much lower frequencies in African-Americans with CD[55]. NOD2 variants have been associated with ileal (or ileocolonic) involvement and stricturing disease[45-47,49,52,56,57]. In most of these studies, homozygous and compound heterozygous patients had increased risk of ileum-specific disease[45], stricturing disease[47,52], or both. Of the known NOD2 variants, the frameshift variant has the strongest association with ileum-specific disease[45] and stricturing disease[52].

BREASTFEEDING

The relationship between breastfeeding in infancy and subsequent development of IBD was first evaluated in the early 1960's by investigators who had observed that some patients with UC demonstrated a striking clinical relationship based on inclusion or exclusion of dairy products from their diet[58]. They conducted a case-control study of 132 adults with UC and 129 controls matched for age and sex. Patients with UC were more likely than controls to never have been breast-fed (χ^2 = 7.42, $0.001 < P < 0.01$) and to have been breast-fed 14 d or less (χ^2 = 9.05, $0.001 < P < 0.005$). Another group of investigators conducted a similar but smaller study with controls matched by age and sex to each of 51 adults with UC and 57 adults with CD. They found that UC patients were more likely never to have been breast-fed than controls (15/51 *vs* 12/102, $P = 0.005$), but there were no differences between CD patients and controls (11/57 *vs* 22/114, P = NS)[18]. However, a population-based case-control study in Sweden demonstrated a significantly shorter duration of breastfeeding in CD patients than in controls matched for sex and age (4.59 mo *vs* 5.76 mo, $P < 0.01$, n = 308 pairs)[59].

One group of investigators conducted two separate studies comparing infant feeding practices among children with IBD and their unaffected siblings. Compared to their unaffected siblings (n = 180), CD patients (n = 114) were less likely to have been breastfed [RR = 3.6 (95% CI 1.4-9.0), $P < 0.01$] and more likely to have received formula food from birth [RR = 3.1 (95% CI 1.3-7.4), $P < 0.02$]. CD patients were younger than their unaffected siblings ($P < 0.01$) but did not differ in gender, birth order, birth month, premature delivery, type of milk used for bottle feeding, age at introduction of solid foods, and length of exclusive and total length of breastfeeding. Multivariate analysis showed that only lack of breastfeeding and diarrheal diseases during infancy were independently associated with later development of CD[19]. In the second study, lack of breastfeeding did not differ significantly between UC patients (n = 93) and unaffected siblings (n = 138) [RR = 1.7 (95% CI 0.77-3.65), P = 0.19]. Multivariate analysis showed that children with UC were more likely than their unaffected siblings to be female (P = 0.01). UC patients and their unaffected siblings did not differ in age, duration of exclusive breastfeeding, total duration of breastfeeding, age at introduction of solid foods, birth order, or premature delivery[20].

A clinic-based pediatric study of 68 CD patients, 39 UC patients and 202 controls, demonstrated a protective effect of breastfeeding on development of CD [breastfeeding $\leqslant$ 5, 6-11, $\geqslant$ 12 mo *vs* not breastfeeding ψ 0.7 (0.3-1.5), 0.6 (0.2-1.5), 0.1 (0.01-1.10), respectively (P_{trend} = 0.04)], and a tendency toward a protective effect of breastfeeding on development of UC [breastfeeding $\leqslant$ 5, 6-11, $\geqslant$ 12 mo *vs* not breastfeeding ψ 0.7 (0.3-1.6), 0.5 (0.2-1.5), 0.2 (0.03-2.20), respectively (P_{trend} = 0.07)][25]. Both associations were controlled for maternal smoking. An Italian multi-center study of incident cases (594 UC patients and 225 CD patients) and randomly selected age and gender matched controls (patients with acute disease not related to smoking, OC use, or immunological disorders) showed an increased risk of IBD in those who had not been breastfed compared to those who had [UC ψ = 1.5 (95% CI 1.1-2.1) n = 594 pairs; CD ψ = 1.9 (95% CI 1.1-3.3) n = 225 pairs]. An increased risk of IBD was detected in subjects who had not been breastfed (controlling for smoking status and OC use), but was statistically significant only in females [UC ψ = 2.2 (95% CI 1.2-3.6) n = 240 pairs; CD ψ = 2.5 (95% CI 1.0-4.9) n = 106 pairs][14]. A Japanese study identified incident cases of IBD in children under the age of 15 years from a national epidemiological survey conducted from 1978 to 1993[60]. Healthy controls were matched to cases by age, sex, and block of birth. Children with CD were significantly less likely to have been breastfed during the first 4 mo of life than were healthy children [ψ = 0.3 (95% CI 0.13-0.70) n = 42 cases, 126 controls]. Children with UC were significantly less likely to have been breastfed during the first 4 mo of life than were healthy children [ψ = 0.53 (95% CI 0.31-0.89) n = 133 cases, 266 controls].

Quite a few smaller studies have evaluated the relationship between breastfeeding and development of IBD. Several of these studies showed a trend toward a protective effect of breastfeeding on the development of IBD, but were too small to achieve statistical significance[16,22,26,61]. Three studies conducted as postal questionnaires all showed no association between breastfeeding and either CD or UC[24,62,63]. These postal questionnaire studies in which cases identified their own controls may have suffered from selection bias[24,62,63] and one of these studies had a very poor response rate thus creating a potential for non-respondent bias[63]. Two large studies failed to demonstrate any differences between cases and controls with respect to breastfeeding in infancy[21,23]. Breastfeeding data for one of these studies was limited to that which was obtained from the hospital chart at the time of the child's delivery thus creating potential for differential misclassification bias[21]. Many of these studies that did not demonstrate a protective effect of breastfeeding on the development of IBD did not characterize breastfeeding as exclusive or mixed and did

not report the duration of breastfeeding. Furthermore, many of these studies did not include potential confounders of the relationship between breastfeeding and IBD. These confounders include family history of IBD, cigarette smoking, OC use, preceding infections, antibiotic use, and various perinatal factors.

Recently, a meta-analysis of all these studies was conducted[64]. Studies were graded based on predefined guidelines. Criteria for the highest grade included recruitment of cases and controls by the investigators, confirmation of diagnosis by a physician, confirmation of breastfeeding information by subjects' mothers or other close relatives, and response rate of at least 80% for both cases and controls. Only four studies received the highest grade for CD[19,21,25,59] and four for UC[20,21,25,58]. Based on all studies of the relationship between breastfeeding and IBD, there was a protective effect of breastfeeding on both CD [ψ_{pooled} = 0.67 (95% CI 0.52-0.86) $P < 0.001$ (heterogeneity test)] and UC [ψ_{pooled} = 0.77 (95% CI 0.61-0.96) P = 0.004 (heterogeneity test)]. Based on the highest grade of studies, the effect of breastfeeding was even more pronounced for both CD [ψ_{pooled} = 0.45 (95% CI 0.26-0.79) P = 0.063 (heterogeneity test)] and UC [ψ_{pooled} = 0.56 (95% CI 0.38-0.81) P = 0.268 (heterogeneity test)][65]. The investigators concluded that their meta-analysis supported the hypothesis that breastfeeding is protective for both CD and UC and that the actual effect is probably greater than their analysis demonstrated due to nondifferential misclassification in some of the studies analyzed.

Subsequently, a population-based, pediatric matched case-control study of environmental risk factors and development of IBD was conducted in Northern France[66]. All IBD cases diagnosed between 1988 and 1997 who were under 17 years of age and resident in the study area at the time of diagnosis were recruited for the study. Randomly selected controls were matched to cases by age, sex, and living area. Subjects were interviewed by trained interviewers and answers were validated using the mandatory child health booklet. Controlling for maternal education level, breastfeeding was an independent risk factor for CD [ψ = 2.1 (95% CI 1.3-3.4) P = 0.003, n = 222 pairs] as were family history of IBD, history of eczema, and BCG vaccination[66]. Drinking tap water (*vs* bottled water or well water) was a protective factor for CD. Regarding the unexpected finding of breastfeeding as a risk factor for CD, the investigators speculated that this association might be the result of either delayed infections at weaning or environmental contamination of the breast milk in the highly industrialized region in which the study was conducted. In the same study, there was no association between breastfeeding and development of UC. Controlling for maternal education, risk factors for UC included family history of IBD, disease during pregnancy, and bedroom sharing, but appendectomy was a protective factor for UC[66].

After publication of this case-control study[66], which met the criteria for the highest grade, the meta-analysis was repeated[64]. Including this study, the protective effect of breastfeeding on the development of CD was diminished [ψ_{MH} = 0.62 (95% CI 0.27-1.43)], but the protective effect of breastfeeding on the development of UC was not altered significantly [ψ_{MH} = 0.62 (95% CI 0.43-0.91)]. More importantly, the inclusion of the most recent study resulted in a much higher heterogeneity for the CD studies ($P < 0.001$, chi-square heterogeneity test). The investigators offered several possible explanations for the surprising different results of the highest quality studies. These included differences in genetic characteristics of the studies' populations, subtypes of CD with different etiologies, and variations in the components of breast milk in the different regions studied[64].

SUMMARY

Despite extensive investigation, the etiology of IBD is still unknown. Clearly, a genetic predisposition to IBD exists[28-30]. Genome-wide scanning studies have identified a series of IBD susceptibility loci, some of which are more strongly associated with CD, others with UC, and some with both[38-40]. Three separate mutations in the *NOD2* gene have been identified that confer susceptibility to CD[41,43], but no specific mutations that confer susceptibility to UC have yet been identified. Despite the strong evidence of genetic predisposition to IBD, it is clear that environmental factors also influence the development of IBD. However, only cigarette smoking has a well established association with IBD. Paradoxically, cigarette smoking is a risk factor for CD, but a protective factor for UC[12]. OC use may also play a role in the etiology of CD although, obviously, only in women[13,14]. Numerous dietary components may play a role in the etiology of IBD although these associations are less certain[16,17]. Many investigators have identified associations between preceding infections and the development of IBD[13,18-20,23,24] and some have identified associations between antibiotic use and development of IBD[23,24]. Many perinatal factors have been studied, but no consistent findings have been reported[21-23,25-27]. Although there have been conflicting reports, meta-analysis of these reports indicates that breastfeeding is a protective factor for both CD and UC[65].

Human breast milk contains many substances that may influence growth and development as well as function of the gastrointestinal tract. Some of these factors may have age-dependent effects[67]. Furthermore, the composition of colonic flora differs between breastfed and bottle-fed infants[68]. IBD pathogenesis is presumed to be a complex interaction between genetic predisposition and inappropriate activation of the mucosal immune system driven by the presence of enteric flora and resulting in tissue injury[1-3]. Thus, it seems quite plausible that breastfeeding would have a protective effect on the development of IBD in genetically predisposed individuals, at least in childhood.

The preponderance of evidence suggests that breastfeeding is a protective factor for IBD, with a greater effect for CD than UC[14,18,19,22,25,58-60]. A meta-analysis of all available studies, taking into account the design of the studies, demonstrated this protective effect of breastfeeding on the development of IBD[65]. However,

this relationship has become more tenuous following the most recent study of the relationship between breastfeeding and IBD[64,66]. Why some studies showed a protective effect of breastfeeding, some showed no effect, and two showed that breastfeeding is a risk factor for IBD is unclear. Proposed explanations include differences in genetic characteristics of the populations studied, subtypes of CD with different etiologies, and variations in the components of breast milk in the different regions studied[64]. The heterogeneous findings may also result from differences in study design. Specifically, the heterogeneous findings may be due to the failure to control for genetic predisposition. Since IBD is thought to occur in genetically predisposed hosts, inclusion of subjects whose genetic predisposition is unknown may be inappropriate. Since estimates of the frequency of NOD2 variants in the Caucasian population range from 4% to 20%[37,43,44,69], inclusion of general population controls may confound results of the investigation.

To date, no studies of the relationship between breastfeeding and IBD have incorporated genetic predisposition into the study design. Two studies have been conducted using unaffected siblings of cases as controls[20,21]. The first of these studies found that children with CD were less likely to have been breastfed [RR = 3.6 (95% CI 1.4-9.0) $P < 0.01$] than their unaffected siblings[19]. This is the strongest association between breastfeeding and CD in any of the published studies. The second study found that children with UC were less likely to have been breastfed than their unaffected siblings but the difference was not statistically significant[20]. Genetic predisposition is important in the etiology of both CD and, to a lesser degree, UC. These two studies were completed long before the discovery of the NOD2 variants that confer susceptibility to CD[41,43] and no susceptibility genes for UC have yet been identified. Nevertheless, these two studies may better reflect the true relationship between breastfeeding in infancy and the subsequent development of IBD than any of the other published studies. To better elucidate the relationship between breastfeeding, or any environmental factor, and the development of IBD, future studies should be conducted in such a way that genetic susceptibility to IBD is considered. Specifically, future studies of the etiology of IBD should be designed such that both environmental factors and genetic factors are incorporated in the same study and gene-environment interaction should be assessed.

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REVIEW

Liver cirrhosis and diabetes: Risk factors, pathophysiology, clinical implications and management

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Abstract

About 30% of patients with cirrhosis have diabetes mellitus (DM). Nowadays, it is a matter for debate whether type 2 DM in the absence of obesity and hypertriglyceridemia may be a risk factor for chronic liver disease. DM, which develops as a complication of cirrhosis, is known as "hepatogenous diabetes". Insulin resistance in muscular and adipose tissues and hyperinsulinemia seem to be the pathophysiologic bases of diabetes in liver disease. An impaired response of the islet β-cells of the pancreas and hepatic insulin resistance are also contributory factors. Non-alcoholic fatty liver disease, alcoholic cirrhosis, chronic hepatitis C (CHC) and hemochromatosis are more frequently associated with DM. Insulin resistance increases the failure of the response to treatment in patients with CHC and enhances progression of fibrosis. DM in cirrhotic patients may be subclinical. Hepatogenous diabetes is clinically different from that of type 2 DM, since it is less frequently associated with microangiopathy and patients more frequently suffer complications of cirrhosis. DM increases the mortality of cirrhotic patients. Treatment of the diabetes is complex due to liver damage and hepatotoxicity of oral hypoglycemic drugs. This manuscript will review evidence that exists in relation to: type 2 DM alone or as part of the metabolic syndrome in the development of liver disease; factors involved in the genesis of hepatogenous diabetes; the impact of DM on the clinical outcome of liver disease; the management of DM in cirrhotic patients and the role of DM as a risk factor for the occurrence and exacerbation of hepatocellular carcinoma.

INTRODUCTION

Up to 96% of patients with cirrhosis may be glucose intolerant and 30% may be clinically diabetic[1]. Currently, it is a matter for debate whether type 2 diabetes mellitus (DM), in the absence of other risk factors contributing to metabolic syndrome (obesity and hypertriglyceridemia), could be a risk factor for the development and progression of liver disease[2-4]. On the other hand, the diabetes which develops as a complication of cirrhosis is known as "hepatogenous diabetes" and is not recognized by the American Diabetes Association and the World Health Organization as a specific independent entity[5].

The liver has an important role in carbohydrate metabolism since it is responsible for the balance of blood glucose levels by means of glycogenogenesis and glycogenolysis[5-11]. In the presence of hepatic disease, the metabolic homeostasis of glucose is impaired as a result of disorders such as insulin resistance, glucose intolerance and diabetes[6,8,11,12]. Insulin resistance occurs not only in muscular tissue, but also in adipose tissue[13], and this combined with hyperinsulinemia seem to be important pathophysiologic bases of diabetes in liver disease[1,3,5,6,14-17]. Additionally, the etiology of liver disease is important in the incidence of DM, since non-alcoholic fatty liver disease (NAFLD), alcohol, hepatitis C virus (HCV) and hemochromatosis are frequently associated with DM[1-3,7,18].

DM in patients with compensated liver cirrhosis may be subclinical, since fasting serum glucose levels may be normal. In these cases, it is necessary to perform an oral

glucose tolerance test (OGTT) to detect an impairment of glucose metabolism[19]. The natural history of hepatogenous diabetes is different from that of hereditary type 2 DM, since it is less frequently associated with microangiopathy. In contrast, the patient with cirrhosis and diabetes suffers more frequently from complications of cirrhosis, which can cause death[2,4,19].

Treatment of diabetes in the cirrhotic patient is complex because of the presence of liver damage and the hepatotoxicity of oral hypoglycemic drugs. Therefore, pharmacological therapy must be closely monitored for the risk of hypoglycemia[3,5,19].

This review will present evidence that exists in the literature in relation to: (1) type 2 DM alone or as part of the metabolic syndrome in the development of liver disease; (2) factors involved in the genesis of hepatogenous diabetes; (3) the impact of DM on the clinical outcome of liver disease; (4) the management of DM in cirrhotic patients. Similarly, we will review the role of type 2 DM and hepatogenous diabetes as risk factors for the occurrence and exacerbation of hepatocellular carcinoma (HCC).

TYPE 2 DM AS A RISK FACTOR FOR NAFLD AND HCC

Epidemiology

Several studies suggest that type 2 DM may have an etiological role in chronic liver disease and HCC regardless of alcohol and viruses[4] (Figure 1).

A total of 173 643 patients with type 2 DM and 650 620 patients without type 2 DM, in whom chronic liver disease was excluded at the time of enrollment and a year later, were observed over a 10-year period in a cohort study. The incidence of non-alcoholic chronic liver disease and HCC was significantly higher in diabetic patients compared with non-diabetic patients. This risk was 2-fold greater and was independent of alcoholic liver disease, viral hepatitis and demographic factors[4]. Although this study has the strength of having a very large number of individuals, it has been criticized because it included a population comprised almost entirely of men (98%) from the Department of Veterans Affairs, and the diagnoses of type 2 DM, chronic liver disease and HCC were taken from a database, and consequently they were not verified biochemically and histopathologically. Additionally, other factors that are part of the metabolic syndrome (which already have a proven influence on the occurrence of NAFLD such as obesity and dyslipidemia) were not taken into account[20].

In a study with a large number of patients carried out in Denmark, the standardized incidence of HCC was higher in men (4.0, 95% CI: 3.5-4.6) and women (2.1, 95% CI: 1.6-2.7) with type 2 DM compared with the general population[21]. Other studies with fewer patients have yielded similar results[22,23].

In a recent case-control study that included 465 patients, DM prevalence was higher in patients with HCC than in controls (31.2% *vs* 12.7%, OR 3.12 95% CI: 2.22-4.43). The DM had been diagnosed prior to the occurrence of HCC in 84% of cases with an average duration of 181.4 mo indicating that it was type 2 DM in most cases[24]. The above data suggests that type 2 DM itself might be a risk factor for the occurrence of HCC. Other studies showed that in the presence HCV, liver fibrosis and alcohol, the risk is higher. Recently, it was observed that patients suffering from chronic hepatitis C (CHC), DM and advanced fibrosis had a 3-fold greater risk than non-diabetic patients with mild to moderate fibrosis of developing HCC in 5 years of follow-up (13% *vs* 5%)[25].

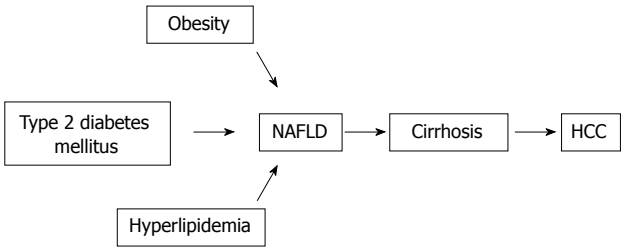

Figure 1 Type 2 diabetes mellitus may give raise to non-alcoholic fatty liver disease (NAFLD) which could progress to cirrhosis and hepatocellular carcinoma (HCC).

NAFLD

NAFLD comprises a series of liver disorders such as simple steatosis, steatohepatitis, fibrosis and cirrhosis. It is estimated that one third of American adult individuals may suffer from fatty liver[26], which is considered the most benign manifestation of NAFLD. The primary fatty liver results from the accumulation of fat, mainly of triglycerides in liver cells in the presence of insulin resistance, and frequently occurs as part of the metabolic syndrome which is made up of obesity, type 2 DM and dyslipidemia[27]. Non-alcoholic steatohepatitis (NASH) is a severe manifestation of NAFLD, since it causes not only steatosis, but tissue inflammation, cell damage and fibrosis. Nevertheless, the prevalence of NASH is estimated at 2%-3%. NASH is regarded as an entity that can progress to cirrhosis and liver failure, and currently it is estimated to be the most common cause of cryptogenic cirrhosis[28,29].

Pathophysiology

The mechanisms by which type 2 DM might cause NAFLD are complex and have been studied in a fragmented manner mainly in isolated biological systems.

It has been observed that the fatty liver, obesity and insulin resistance act as co-factors to cause liver damage[1,3,4]. Fatty liver is the result of an intracellular accumulation of triglycerides because of increased uptake of free fatty acids and *de novo* liponeogenesis in the hepatocytes. At the same time, there is a reduction in the hepatic secretion of very low density lipoproteins. The liver damage consists of cellular necrosis and inflammation, and these disorders result from an increase in mitochondrial oxidative stress on triglycerides with the consequential generation of free radicals and peroxisomes[30,31]. The mitochondrial oxidative

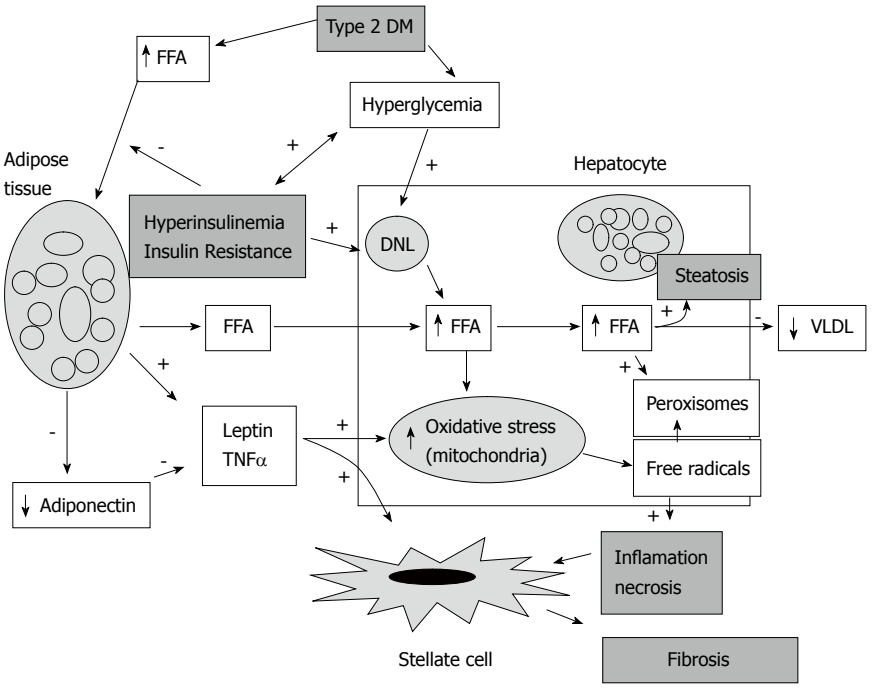

Figure 2 Liver damage caused by type 2 DM. Insulin resistance promotes release of free fatty acids (FFA) from adipose tissue. The FFAs are accumulated in the liver cells, and *de novo* liponeogenesis (DNL) contributes also. The reduced secretion of very low density lipoprotein (VLDL) by hepatic cells saturates hepatocytes producing steatosis. Mitochondrial oxidative stress is increased as a result of excess intracellular FFAs and the influence of adipokines (leptin and tumor necrosis factor alpha (TNF-α). Excess of oxidative stress produces free radicals which in turn induces inflammation and cellular necrosis. Tissue inflammation stimulates the stellate cells to produce collagen.

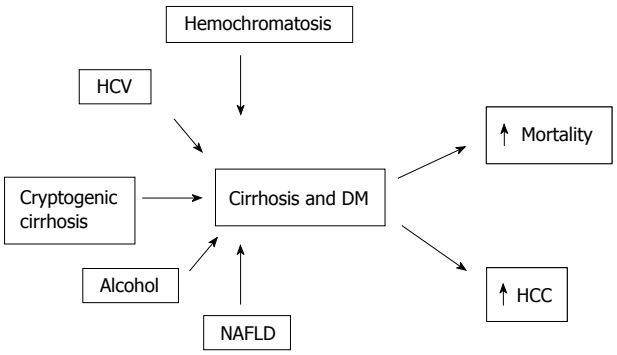

Figure 3 Etiology of liver cirrhosis most frequently associated with diabetes mellitus.

stress is increased also by the action of adipokines (cytokines produced by the adipocytes) such as leptin and tumor necrosis factor-α (TNF-α), which are produced in excess[32]. The reduction of adiponectin, which is a regulatory adipokine, favors the activity of inflammatory adipokines[33]. These chemical mediators, derived from inflammation and cell necrosis, as well as the adipokines activate the liver stellate cells and induce them to increase production of collagen, connective tissue growth factor and accumulation of extracellular matrix, in turn favoring fibrosis[34] (Figure 2).

DM AS A COMPLICATION OF CIRRHOSIS-HEPATOGENOUS DIABETES

Epidemiology

Depending on the etiology, the degree of liver damage and the diagnostic criteria, the reported incidence of glucose intolerance varies from 60 to 80% and that of diabetes between 20 and 60%[3,5,16,19]. It is known that from the early stages of chronic liver disease, insulin resistance and glucose intolerance may be found in most of these patients[35,36]. The diabetes manifests clinically as the liver function deteriorates, thus hepatogenous diabetes can be considered as an indicator of advanced liver disease[37].

The etiology of chronic liver disease is crucial in the development of hepatogenous diabetes: alcohol, HCV, hemochromatosis and NASH (Figure 3).

NASH: NASH is a severe manifestation of NAFLD. NASH is associated with visceral obesity, hypertriglyceridemia, and virtually all patients have insulin resistance. Therefore, it is not surprising that type 2 DM is present in 30%-45% of patients with NASH[38].

On the other hand, it has been observed that obesity itself is an independent risk factor for severe liver disease[39]. Obesity is characterized by expanded adipose tissue which is in a state of chronic inflammation resulting in an increase in the secretion of adipokines. These adipose tissue cytokines have a systemic effect particularly on the liver, which leads to an altered metabolic state with insulin resistance, hyperglycemia and hyperinsulinemia; these abnormalities disrupt the liver metabolism of lipids[40]. Cytokines, of which TNF-α is the most studied member, stimulate the liver stellate cells directly inducing hepatic fibrosis[41]. The body weight reduction improves metabolic abnormalities that accompany the metabolic syndrome such as hyperlipidemia and fatty liver[42].

CHC and HCV: In a study conducted by The National Health and Nutrition Examination Survey, a 3-fold higher risk of DM was identified in individuals over 40 years of age with CHC, compared with those patients with non-C chronic hepatitis[43]. Knobler *et al* observed a prevalence of DM of 33% in non-cirrhotic patients with CHC, compared with 5.6% in a control group[44]. In patients chronically infected with HCV, fatty liver was observed in 30%-70% of cases[45].

In patients with CHC, a high prevalence of glycometabolic abnormalities is reported such as glucose intolerance in more than 40% and DM in more than 17%. Additionally, the insulin resistance observed in these patients is an independent risk factor for steatosis in relation to the severity of fibrosis[7,46-48].

The mechanisms by which HCV produces insulin resistance and DM are not clearly known. It has been observed that HCV induces insulin resistance regardless of body mass index and fibrosis stage. In a study conducted in a transgenic animal model, the HCV core protein was able to induce insulin resistance, steatosis and DM. TNF-α overproduction seems to have been the primary mechanism. This cytokine phosphorylates the serine residues of the insulin receptor (IRS-1 and IRS-2), and stimulates the overproduction of suppressor of cytokines (SOC-3). SOC-3 inhibits phosphorylation of Akt and phosphatidylinositol 3-kinase. All these disorders, related to intracellular signaling of insulin, could block the transactivation of GLUT-4, which would result in block of glucose uptake at the cellular level. Indeed, in the transgenic mouse, TNF-α correlates with the hyperinsulinism and TNF-α block occasioned by the administration of anti-TNF-α drugs such as infliximab avoids the appearance of insulin resistance. Therefore, the mechanisms by which HCV induces insulin resistance include: production of TNF-α, serine phosphorylation of IRS and overexpression of SOCs. Furthermore, the overproduction of TNF-α in patients with CHC correlates with a faster progression of fibrosis and a lower response to interferon[40].

On the other hand, HCV genotype may be of importance in the occurrence of glucose metabolic disorders, as genotypes 1 and 4 are significantly associated with insulin resistance more frequently than genotypes 2 and 3 (37% *vs* 17%)[47]. It is well demonstrated that genotypes 1 and 4 are associated with a lower viral sustained response to antiviral therapy than genotypes 2 and 3. Insulin resistance may be a cofactor that increases the failure of the response to antiviral treatment observed in these patients. In accordance with this, in a recent study in patients with HCV genotype 1, those with HOMA > 2 (insulin resistance) had a 2-fold lower sustained response to treatment than patients with HOMA < 2 (32.8% *vs* 60.5%, respectively)[49]. For sustaining this idea, it is important to note that in experiments carried out with Huh-7 cells infected with HCV RNA, viral replication was blocked by adding interferon to the system. However, the ability of interferon to block viral replication was abolished when insulin was added to interferon at a dose of 128 mcU/mL (similar to that seen in the hyperinsulinemic states)[50]. Finally, it has been reported that patients with CHC and insulin resistance have a less sustained response to peginterferon plus ribavirin treatment compared with patients without insulin resistance[40,49].

It seems that once the insulin resistance and DM-inducing mechanisms in CHC are fired, their courses are not affected by the presence or absence of viral activity. Indeed, in a recent study it was observed that HCV clearance by pegylated interferon and ribavirin treatment did not reduce the risk of DM in patients with chronic hepatitis and normal fasting blood glucose during a period of 8 years of follow-up after treatment. Patients with a sustained response had a similar incidence of DM compared with those who did not respond to treatment (14.8% *vs* 18.5%, respectively)[51].

Alcohol: Patients with alcoholic liver disease have a high relative risk of suffering diabetes[52]. This risk is directly related to the amount of ingested alcohol, as it rises 2-fold in patients ingesting more than 270 g of alcohol per week compared with those ingesting less than 120 g/wk[53]. Acute alcohol ingestion produces a significant reduction in insulin-mediated glucose uptake. On the other hand, patients with chronic alcoholism frequently have chronic pancreatic damage and injury of pancreatic islet β-cells resulting in DM[1].

Hemochromatosis: Hereditary hemochromatosis is a disease characterized by iron accumulation in several organs-particularly in the liver - as a result of a disorder of the metabolism of this metal. This abnormality is produced by a mutation of the *HFE* gene. In addition, the iron can infiltrate the pancreas and myocardium. In the pancreas, the concentration of iron is predominantly in the acinus of exocrine secretion. However, infiltration of Langerhans islets with damage to the insulin-producing β-cells can also be observed. This is the reason why DM can be observed in 50%-85% of patients with hereditary hemochromatosis in advanced stages[54]. Additionally, glucose metabolic disorders resulting from the liver damage probably contribute to the high frequency of DM[1,5].

Pathophysiology of hepatogenous diabetes

The pathophysiology of hepatogenous diabetes is complex and not precisely known. Insulin resistance in peripheral tissues (adipose and muscular tissue) plays a central role in the glucose metabolism disturbance[1,2,9,11,14-17]. It has also been proposed that reduced insulin extraction by the damaged liver and portosystemic shunts result in hyperinsulinemia which is potentiated by raised levels of contra-insulin hormones (glucagon, growth hormone, insulin-like growth factor, free fatty acids and cytokines)[2,11,15,17]. However, a recent study reports that in patients with Child B grade liver cirrhosis the hyperinsulinism may be produced by an increase of the pancreatic β-cell sensitivity to glucose, whereas disturbance of hepatic insulin extraction does not seem to have a significant role[55]. It has also been speculated that genetic and environmental factors and some etiologic agents in liver disease such as HCV, alcohol, and iron infiltration impair the insulin secretion activity of the β-cells of the pancreas[9]. In conclusion it seems that glucose intolerance may result from two abnormalities that occur simultaneously: (1) insulin resistance of muscle and (2) an inadequate response of the β-cells to appropriately secrete insulin to overcome the defect in insulin action. On the other hand DM develops as the result of progressive impairment in insulin secretion together with the development of he-

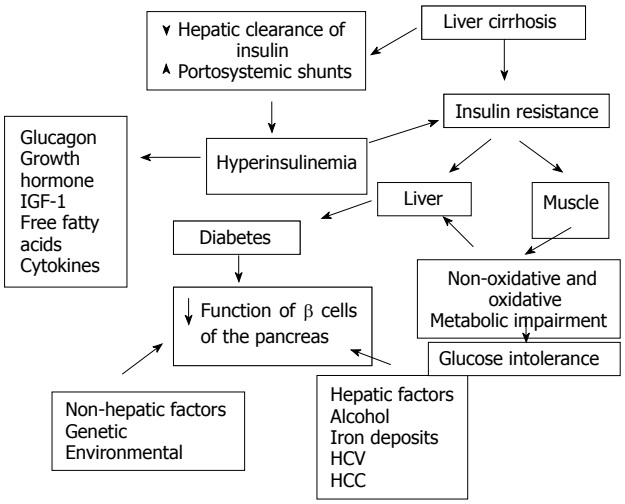

Figure 4 Pathophysiology of hepatogenous diabetes. One of the main abnormalities is insulin resistance in muscular cells and the hepatic tissue. Insulin resistance in muscle impairs non-oxidative and oxidative glucose metabolism. The reduction of insulin clearance by the damaged liver and the presence of portosystemic shunts in one hand and the desensitization of the beta cells of the pancreas produced by diverse factors on the other hand may produce hyperinsulinemia. With progression of the diabetes there is a reduction in sensitivity of β-cells for production of insulin.

patic insulin resistance leading to fasting hyperglycemia and a diabetic glucose tolerance profile[14,15] (Figure 4).

Discrimination between hepatogenous diabetes and type 2 DM may be difficult. In a recent study comparing patients with hepatogenous diabetes *vs* patients with type 2 DM, the ratios of postprandial plasma glucose (PP2h) to fasting plasma glucose (FPG) (2.27 *vs* 1.69), fasting insulin (23.2 *vs* 11.6 microIU/mL) and HOMA-Insulin Resistance index (8.38 *vs* 3.52) were significantly higher in patients with hepatogenous diabetes. Therefore, insulin resistance in liver cirrhosis is higher than in type 2 DM, and impairment of hepatic insulin degradation may be an important mechanism of hyperinsulinemia in liver cirrhosis[56].

TYPE 2 DM AND HEPATOGENOUS DIABETES AGGRAVATE LIVER CIRRHOSIS AND HCC

DM increases morbidity and mortality of liver cirrhosis patients

The effect of type 2 DM and hepatogenous diabetes on the clinical outcome of cirrhosis and HCC has been evaluated in only few studies. In cross-sectional retrospective studies in patients with cirrhosis of any etiology it has been observed that the DM is associated with an increased risk of complications[5,38,57,58]. According to the Verona study, which is based on a population of more than 7000 individuals suffering from type 2 DM, the risk of death at 5 years was 2.52-fold greater (CI 1.96-3.2) than in the general population[59]. Other studies report that DM, obesity and steatosis are associated with liver disease and more severe fibrosis in CHC[60,61].

It is important to note that neither the Child-Pugh nor Model for End-Stage Liver Disease (MELD) Scores (which are widely used as prognostic instruments of morbidity and mortality in the short and long term for cirrhotic patients) include in their parameters DM or glucose intolerance[62,63]. Nevertheless, interesting data have been observed in some prospective longitudinal studies involving cirrhotic patients where DM has been studied as an independent prognostic factor. In a retrospective and prospective study 354 (98 with diabetes) of 382 eligible patients were followed for 6 years after inclusion into the study: 110 were alive at the end of follow-up. Prognostic factors identified by Kaplan-Meier analysis, followed by Cox's stepwise regression demonstrated in sequence, albumin, ascites, age, encephalopathy, bilirubin, diabetes, and platelets as prognostic factors of mortality. The larger mortality rate in patients with diabetes, was not due to complications of diabetes but to an increased risk of hepatocellular failure[58]. Diabetes was no longer a risk factor as a covariate in a subgroup of 271 patients when varices were added but was again significant when patients who died of gastrointestinal bleeding were excluded.

In another study carried out in patients suffering from cirrhosis and refractory ascites on the waiting list for liver transplantation it was observed that the HCC and DM, but not the Child-Pugh score, were independent predictive factors of mortality. The patients suffering from refractory ascites and DM showed a 1- and 2-year probability of survival of 32% and 18%, respectively. By contrast survival rates of patients with refractory ascites without DM were 62% and 58%, respectively[64].

Nishida *et al* performed the OGTT on a group of 56 patients with cirrhosis and normal fasting blood glucose. A total of 38% of patients were diagnosed with DM, 23% with glucose intolerance, and 39% were normal. After 5 years of follow-up, patients with diabetes and glucose intolerance had significantly higher mortality than normal patients (44% and 32% *vs* 5%, respectively). From a multiple regression analysis only serum albumin and DM were independent negative predictive factors of survival[19].

Hepatogenous diabetes has a clinical behavior different from that of hereditary type 2 DM, since it is less frequently associated with retinopathy and cardiovascular and renal complications[5,58]. In cirrhotic patients with diabetes, the most recurrent cause of death is liver failure[4,19,58].

DM increases the severity and mortality of HCC

At present, type 2 DM is considered a risk factor for the occurrence of HCC. Hepatogenous diabetes together with hepatitis B and C virus infection and alcoholic liver cirrhosis increases the risk of HCC by 10-fold[1,2].

Patients with HCC and DM have a mortality risk higher than patients with HCC without DM. In another study involving 160 patients suffering from HCC, those who had DM had a 1-year mortality rate higher than those patients without DM. Additionally, they had more extensive disease[65].

Pathophysiologic mechanisms

The mechanisms by which diabetes worsens the clinical course of liver cirrhosis have not been clearly established. Firstly, DM accelerates liver fibrosis and inflammation

giving rise to more severe liver failure. Secondly, DM may potentiate the incidence of bacterial infections in cirrhotic patients which are associated with increased mortality[66,67].

In relation to the first mechanism, insulin resistance increases adipokine production (cytokines secreted by adipose tissue), such as leptin and TNF-α, which activates the inflammatory pathways that exacerbate liver damage[68]. In contrast, another cytokine produced by adipose tissue, adiponectin, is a regulator of insulin sensitivity and tissue inflammation[69]. A reduction in the adiponectin levels reflects peripheral and hepatic insulin resistance[70]. There has been speculation that hypoadiponectinemia may play a role in liver disease progression[70,71].

Regarding the second mechanism, DM may worsen immunodepression in cirrhotic patients thus increasing the incidence of severe infections which may have deleterious effect on liver function. It should be noted that cirrhotic patients with spontaneous bacterial peritonitis have a high hospital mortality rate due to sepsis, liver failure and hepatorenal syndrome. On the other hand, patients with esophageal variceal bleeding have a high incidence of infections that increase their in-hospital mortality rate[72]. Notwithstanding, it has not been established if DM increases the mortality rate in patients with other complications of cirrhosis.

In future, the precise mechanisms by which DM may worsen liver function should be clarified, since manipulation of these may be useful for reduction of complications.

CLINICAL IMPLICATIONS OF DM IN THE COURSE OF LIVER CIRRHOSIS AND TREATMENT OF DIABETES

Clinical manifestations

Clinical manifestations of DM in the early stages of cirrhosis are virtually absent. In a recently published study involving compensated cirrhotic patients with normal fasting serum glucose and without a family history of type 2 DM, up to 77% had DM or glucose intolerance diagnosed by means of OGTT. In 38% of cases, DM was subclinical[19]. As liver function deteriorates, the incidence of diabetes increases so that clinical diabetes may be seen as a marker of liver failure.

Hepatogenous diabetes has particular clinical characteristics: (1) unlike the hereditary type 2 DM, it is less frequently associated with risk factors such as age, body mass index, and family history of diabetes; (2) it is less frequently associated with retinopathy and cardiovascular and renal complications; (3) it is more frequently associated with hypoglycemic episodes as a result of impaired liver function[2,19].

Although the incidence of obesity, DM and metabolic syndrome have increased in the world, reaching epidemic proportions, the role of DM as a prognostic factor of morbidity and mortality in cirrhotic patients has been scarcely studied. In addition, the impact of early diagnosis and treatment of DM on the clinical course of cirrhosis is unknown.

Treatment

Few studies have evaluated what is the most efficacious therapy for DM in cirrhotic patients and what is the impact of treatment of DM on the clinical course of liver disease.

The treatment of DM of cirrhotic patients has particular characteristics that make it different from type 2 DM without liver disease: (1) about half the patients have malnutrition; (2) when clinical DM is diagnosed, the patient has advanced liver disease; (3) most of the oral hypoglycemic agents are metabolized in the liver; (4) patients often have episodes of hypoglycemia[3].

The initial treatment of patients suffering from mild to moderate hyperglycemia and compensated liver disease may be a lifestyle change, since at this stage the insulin resistance is a dominant factor. However, these therapeutic measures may be compromised by very restrictive diets, since they might aggravate malnutrition in some patients. On the other hand, physical exercise which improves insulin resistance may not be an appropriate measure in patients with active liver disease[15].

When DM manifests in advanced stages of liver disease, the use of oral hypoglycemic drugs may be required. However, most of these drugs are metabolized in the liver, therefore, the blood glucose levels during treatment shall be closely monitored in order to avoid hypoglycemia[73]. In these cases, biguanides, which reduce resistance to insulin, may be useful. Metformin is a biguanide that is relatively contraindicated in patients with advanced liver failure and patients who continue to ingest alcohol, because of the risk of lactic acidosis[74].

On the other hand, insulin secretagogues, despite the fact that they are safe drugs in patients with liver disease, probably are not useful, since they do not modify insulin resistance and patients with alcoholic cirrhosis often have pancreatic islet β-cell damage[75]. These patients have chronic compensatory hyperinsulinemia until the islet β-cells are exhausted.

Alpha-glucosidase inhibitors can be useful in patients suffering from liver cirrhosis, since their mechanism of action is to reduce carbohydrate absorption in the bowel, thus reducing the risk of postprandial hyperglycemia that is common in these patients. In a randomized, double-blind study involving 100 patients with compensated liver cirrhosis and insulin-treated DM, the control of postprandial and fasting blood glucose levels improved significantly with the use of acarbose, an alpha-glucosidase[76]. In another crossover placebo-controlled study involving patients with hepatic encephalopathy, acarbose produced a significant improvement in postprandial blood glucose level. Additionally, the patients had a reduction in plasma ammonia levels and an increase in the frequency of bowel movements[77]. The reduction in ammonia levels was probably the result of a decrease in the proliferation of intestinal proteolytic bacteria caused by bowel movement[77].

Thiazolidines may be particularly useful in cirrhotic patients with DM, since they increase the insulin sensitivity. However, troglitazone has been withdrawn from the market because of its potential hepatotoxic effects.

Nevertheless, rosiglitazone and pioglitazone appear to be safer drugs[78], but it is recommended that these drugs should not be initiated if there is evidence of active liver disease or if alanine transaminase levels are above 2.5 times the upper limit of normal. The use of these drugs should be monitored closely whenever necessary.

Insulin requirements in the cirrhotic patient with diabetes may vary. In patients with compensated cirrhosis, requirements may be greater compared to patients with decompensated cirrhosis, since insulin resistance predominates in the former while in the latter liver metabolism of insulin is greatly reduced. Therefore, therapy with insulin must be preferably performed in hospitalized patients with close monitoring of blood glucose levels for development of hypoglycemia[79].

Finally, liver transplantation rapidly normalizes glucose tolerance and insulin sensitivity. It is thought that this effect is due to an improvement in the hepatic clearance and peripheral glucose disposal. The latter effect could be secondary to a correction of chronic hyperinsulinemia[16,80]. It has been observed that liver transplantation, in reducing insulin resistance, cures hepatogenous diabetes in 67% of cirrhotic-diabetic patients. In 33% of patients diabetes was not corrected because of persistence of a reduced β-cell function measured by means of an OGTT. This abnormality would make these patients eventually eligible for combined islet transplantation[81].

FUTURE PERSPECTIVES IN RESEARCH OF DM IN LIVER CIRRHOSIS

Future research in the field of DM in cirrhotic patients should clarify the following issues: (1) the role of isolated type 2 DM in the genesis of chronic liver disease and the factors involved in this complication; (2) the impact of hepatogenous diabetes in the natural history of cirrhotic patients; (3) the impact of early diagnosis and treatment of hepatogenous diabetes (through OGTT) in reducing mortality; (4) the benefits of controlling the DM in the management of complications of liver cirrhosis; (5) the mechanisms by which hepatogenous diabetes increases morbidity and mortality of cirrhotic patients, as well as the impact of the manipulation of these mechanisms on the patients; (6) the establishment of clearer guidelines for management of diabetes in the cirrhotic patient.

Perhaps the combination of DM with the currently used scores (Child-Pugh and MELD scores) may enhance the sensitivity and the specificity for prediction of morbidity and mortality rates in cirrhotic patients.

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TOPIC HIGHLIGHT

Simon D Taylor-Robinson, MD, *Series Editor*

Contrast-enhanced 3D ultrasound in the radiofrequency ablation of liver tumors

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Abstract

Liver metastases and hepatocellular carcinomas are two of the most common causes of cancer deaths in the world. Radiofrequency ablation (RFA) is a well recognized, effective and minimally invasive means of treating malignant hepatic tumors. This article describes the use of contrast-enhanced 3D ultrasound (CE-3DUS) in the staging, targeting and follow-up of patients with liver tumors undergoing RFA. In particular, its value in the management of large hepatic lesions will be illustrated. Current limitations of CE-3DUS and future developments in the technique will also be discussed. In summary, CE-3DUS is useful in the RFA of liver tumors with improved detection and display of occult lesions and recurrence, in the assessment of lesional geometry and orientation for a more accurate planning and guidance of multiple RFA needle electrodes in large tumors and in the evaluation of residual or recurrent disease within the immediate and/or subsequent follow-up periods.

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Key words: Liver tumors; Radiofrequency ablation; Contrast enhanced 3D ultrasound

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INTRODUCTION

Colorectal cancer liver metastases and hepatocellular carcinomas are the two most common malignancies of the liver, associated with a dismal outcome of zero survival at 5-year if left untreated. Radiofrequency ablation (RFA) is rapidly emerging as one of the most popular, effective, minimally invasive, alternative therapeutic tool to hepatic surgery in the treatment of these liver malignancies. Conventional 2-dimensional (2D) unenhanced ultrasound (US) guidance of RFA is limited due to its lower sensitivity when compared with the referal modalities, such as contrast enhanced computerized tomography (CT) or magnetic resonance imaging (MRI). Recent advances in non-linear imaging modes and developments in 3-dimensional (3D) mechanical and electronic ultrasound probes have led to a marked improvement in the real-time contrast enhanced volumetric imaging with potential impact in the detection, planning and targeting strategy of RFA needle electrodes in the treatment beyond small lesions. The objective of this article is to demonstrate the usefulness of contrast-enhanced 3D ultrasound (CE-3DUS) in the radiofrequency ablation of these liver tumors. The indications for RFA of metastases and hepatocellular carcinomas will be reviewed within the appropriate clinical settings. The applications of CE-3DUS in all the aspects of staging, planning, targeting and follow-up of RFA are described and illustrated.

CLINICAL BACKGROUND

In the Western World, colorectal cancer accounts for

14 and 16 percent of cancer deaths in men and women, respectively, with approximately 25% of patients having liver involvement at the time of initial presentation and up to 50% will develop hepatic metastases during the course of their disease[1,2]. For patients with colorectal liver metastases, surgical resection is the treatment of choice, but only 10%-20% of patients are initially candidates for potentially curative resection; resection should be considered if there is no unresectable extra-hepatic disease, all liver deposits can be resected with a free clearance margin of 1 cm, and there is adequate liver reserve. The five-year survival rates vary from 25%-40%[3,4]. Seventy five percent of those who undergo liver resection will develop recurrence and of these, the liver is involved in 50%. Sixty five to eighty five percent of all recurrences appear within the first 2 years[5]. Repeat liver resection in these patients still has a 5-year survival of 30 to 40 percent. Whilst the post-operative mortality or morbidity following repeated hepatectomy is comparable to those of single hepatectomy, they are not entirely negligible and hepatectomy is associated with significant monetary expense[5,6].

The worldwide incidence of hepatocellular carcinoma is increasing and in particular within North America and Europe, which is progressively affecting younger patients. It is now believed that this is mainly attributed to the rise in hepatitis C viral infection, as the rates associated with alcoholic cirrhosis and hepatitis B virus infection have remained stable[7]. The disease is extremely lethal with median survival rates of untreated symptomatic cases ranging between 4 to 6 mo. Patients with even small tumors also carry a significant mortality as less than 50% will survive 5 years despite undergoing apparently curative resection. Patients with early stage hepatocellular carcinoma should be offered surgical therapeutic options of transplantation or resection[8]. Transplantation offers a 4-year overall survival rate of 75% and a 4-year recurrence free survival rate of 83%. However, few would benefit from transplantation given the shortage of living donors and the eligibility of patients to the "Milan criteria" for transplantation (i.e. decompensated cirrhosis, solitary tumor smaller than 5 cm and up to 3 lesions smaller than 3 cm)[9]. Similarly, less than 5% of cirrhotic patients with hepatocellular carcinoma would be suitable for hepatic resection under current criteria (those with limited tumour burden and relatively well preserved liver function).

Given the shortcomings of current surgical approach, an effective, minimally invasive and repeatable technique for the treatment of liver tumors could potentially impact favorably in the management of these patients.

RADIOFREQUENCY ABLATION

During the last decade, there has been considerable development of the ablative techniques for oncological applications including cryo-, radiofrequency-, microwave- or laser- ablation and high intensity focused ultrasound (HIFU). The development of radiofrequency ablation can be traced back to 1891 through the works of d'Arsonval[10]. In more recent years, with the additional refinements to the design and power of the equipments, it has emerged as the most popular tool for the destruction of hepatic as well as other malignant tissues. During the application of radiofrequency energy, a high frequency alternating current moves from the electrode in the immediate surrounding tissue and as the ions within the tissue attempt to follow the change in the direction of the alternating current, frictional heating of the tissue is generated. Tissue temperature can be elevated beyond 100 degrees centigrade, resulting in coagulation necrosis of the tissue. Precise control of the extent of tissue destruction can be achieved by adjusting local temperature and electrical resistance.

Radiofrequency ablation of liver tumors can be performed percutaneously, laparoscopically or as part of an open surgical procedure[11-13]. Ultrasound guidance remains the optimal method for accurate targeting of the tumors with the RFA needle electrode as it is mobile, more practical, readily available, rapid and cost-effective compared with CT or MR guidance. Despite the availability of screening facilities with the current advanced CT or MR modalities, RFA guidance with the latter modalities is limited for multiple lesional ablations during the same session requiring repeated large volumes of contrast administration to complete all imaging requirements; as such there is some hampering of the work flow for the whole ablative process. As a result many centers use a combination of both CT and US for the ablative procedure.

INDICATIONS FOR RADIOFREQUENCY ABLATION

Radiofrequency ablation is indicated in patients with disease limited to the liver who do not meet the criteria for surgical resectability for both hepatocellular carcinomas and liver metastases[14-15]. RFA is now offered to those who cannot undergo resection because of inadequate surgical margins, inadequate liver reserve, co-existing morbidity or patient choice and is performed with a curative intent as in surgical resection. RFA is also effective in the destruction of lesions localized adjacent to major vascular structures including the hepatic veins confluence with the inferior vena cava which would preclude resection. The blood flow in major vessels acts as a heat sink that protects the vascular endothelium from thermal injury whilst allowing complete coagulation of tissue immediately surrounding the blood vessel wall. However, RFA is avoided for peri-hilar lesions due to potential biliary damage leading to fistulous or stricturing complications. With the increasingly aggressive approach adopted by liver surgeons, open radiofrequency ablation is routinely combined with liver resection in the presence of multi-focal, bilateral metastases or upon the detection of unexpected additional lesion. In these cases hepatectomy is performed to deal with the main tumor bulk and any residual tumors that cannot be resected,

is treated with RFA. Nonetheless, standard surgical considerations still apply with no more than 70% of the liver volume is removed and particular attention has to be paid to patients with background liver cirrhosis with limited functional reserve.

The use of new effective systemic chemotherapy for the colorectal liver metastases has increased the potential of obtaining significant response to the point of enabling resectability. Studies have shown that of patients with unresectable disease who received second line neo-adjuvant therapy, up to 22% became resectable[16]. There is also a growing proportion of these patients who have had their disease down-staged, who are subsequently referred for radiofrequency ablation instead of surgical resection. The development of fairly extensive steatosis in 20% to 66% of these patients is among the main reason for the choice of RFA, as there is significant morbidity and mortality associated in these cases following liver resection[17,18]. Patients with large hepatocellular carcinomas are also now being considered for trans-arterial chemo-embolisation/trans-arterial embolization followed by RFA of any residual disease. However, there is as yet no evidence that these combination therapies with RFA leads to any survival benefit compared with standard clinical practice. The use of RFA in the treatment of other types of metastatic tumors to the liver have also been advocated; for example stable (6 mo) disease from breast, renal, melanoma or neuro-endocrine metastases[13,19].

IMAGING TASKS FOR RFA

Intra-operative ultrasonography (IOUS) has been shown to yield significant new information, not identified on pre-operative imaging, which determines resectability or changes the operative plan in up to 50% of patients; it is considered the gold standard thereby achieving universal usage[20-23]. However, traditionally CT and MRI have been used to routinely stage all patients with hepatocellular carcinomas and metastatic hepatic colorectal disease. Given the well-recognized limitations of conventional unenhanced ultrasound, its role in the liver staging process has been negligible. More recently, there has been increasing interest in the use of ultrasound contrast agents during the sonography of the liver to improve the detection of liver metastases. Ultrasound contrast agents consist of microbubbles of air or gases of low solubility, stabilized by a lipid, surfactant or polymer shell. Analogous to CT or MR, it is the relative distribution of the contrast agents between normal tissue and the lesion, which makes the lesion more visible and easier to characterized[24,25]. Recent advances in non-linear imaging in the form of pulse inversion together with power modulation modes, combined with the development of contrast agents with liver specificity, have markedly improved the sensitivity of sonography in the detection of small metastases, which may be equal to or even superior to that of CT or MR in some cases[26,27].

Whilst we are accustomed to viewing cross-sectional imaging in a two dimensional perspective, tumor staging, its treatment planning, targeting and assessment of therapeutic response would clearly benefit from a three dimensional morphological as well as functional imaging aspect. In that respect, as a result of technological innovations in non-linear modes (NLM) and new probe design, real time imaging with CE 3D-US is now a reality.

Liver staging

Almost all patients are referred for RFA, based on the CT and/or MR imaging findings; RFA would only be feasible under ultrasound guidance, if all the lesions identified on the referral imaging modalities could actually be detected on ultrasound itself. However, conventional ultrasound is well recognized to be limited in the detection of liver metastases and in the identification of hepatocellular carcinoma in a multi-nodular cirrhotic background. Furthermore, in patients with colorectal liver metastases who have had neo-adjuvant chemotherapy and subsequently referred for RFA, the difficulty of identifying all metastases is significantly increased due to widespread steatosis. Compared with conventional ultrasound, contrast-enhanced ultrasound (CE-US) has been shown to be highly accurate in determining the extent and distribution of tumor burden within the liver. Recent studies have shown that the sensitivity and accuracy of CE-US are comparable with those of CT and/or MR enhanced with liver specific contrast agents[26,27]. Within the RFA clinical setting, the sensitivity of ultrasound in the detection of metastases, HCCs and all lesions combined has been reported to be 42.9%, 66.7% and 51.4%, respectively; in comparison, the sensitivity for contrast enhanced ultrasound was 100% for all 3 groups[28]. Complete ultrasound guided RFA would have been impossible without the use of contrast agent in 60.4% as the lesions remained occult on the unenhanced ultrasound and of these, no lesions could be detected in 15.4%. To identify all lesions as seen on CT/MRI, contrast enhanced ultrasound was required in 88.9% patients with metastases compared with 41.3% of patients with hepatocellular carcinomas. Of the patients with metastases, 44.4% had been on second line systemic chemotherapy and all required contrast enhanced ultrasound to detect all lesions. No patient was subsequently referred for CT/MRI guided RFA. Follow-up CT/MRI confirmed successful targeting in all patients.

Moreover, in staging the liver for ablation, characterization of all the targeted lesions is important to ensure they are truly malignant; this is particularly so with referrals based on CT scans where hemangiomas may be misdiagnosed as metastases. In patients who have had systemic chemotherapy, there are potential problems with underestimation of the true extent of the disease as well as presence of pseudo-tumors due to diffuse areas of fatty infiltration and focal areas of fatty sparing, respectively. Contrast enhanced ultrasound is particularly useful in confirming the true nature of these benign lesions with real-time evaluation of the lesional micro-vascularization (Figures 1 and 2).

From a practical standpoint, scheduling for CT or

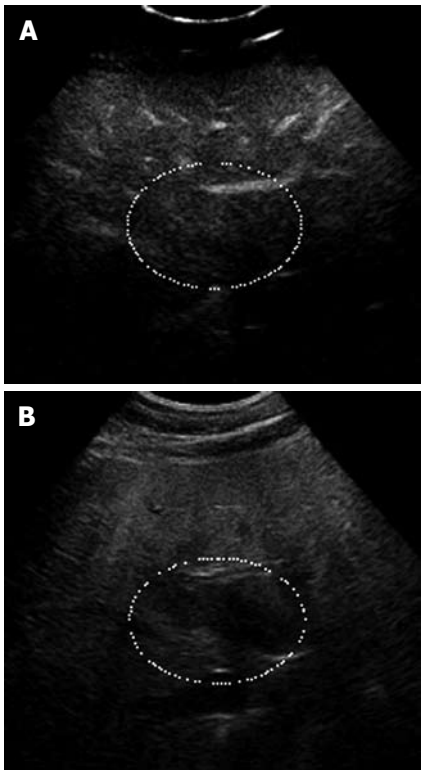

Figure 1 Focal fat sparing shown as iso-echogenicity as adjacent liver parenchyma. A: Late phase of CE-US; B: Hypo-echoic area on the fundamental mode.

MR scans within the 4-6 wk of the RFA procedure may not always be possible because of lack of availability and/or accessibility. With the benefit of its higher accuracy, contrast enhanced ultrasound also offers the flexibility in enabling re-staging immediately prior to the RFA procedure. Using standardized scanning protocols, CE 3D-US may even be superior to CE-US (2D) in the detection and display of these occult liver tumors as well as improving workflow. To ensure complete coverage of the liver, scanning protocols with CE-3DUS include wide angled automated sweeps at the epigastrium, sub-costally and the 3 intercostal spaces in the right upper quadrant of the abdomen, during the arterial, portal venous and late phases of the intravenous bolus injection of the contrast agent. The CE-3DUS set of data may be transferred onto a workstation or a PACS for archiving and reviewed subsequently; it can also be displayed in the same manner as CT or MRI, analyzed and then reported (Figure 3). The whole examination may be performed by the sonologist and the review can be carried out immediately online or subsequently by the sonologist or the Radiologist with improvement in the workflow. There are anecdotal reports of the superiority of CE-3DUS over baseline fundamental unenhanced 2D and 3D ultrasound in the detection and display of occult liver metastases (Figure 3). Furthermore, in dealing with larger tumors and in particular local recurrence, CE-3DUS can define more clearly the biologically active target tumor volume. This is supported by recent preliminary study using a 3D Shape based analysis of CT scans showing that the technique may be useful

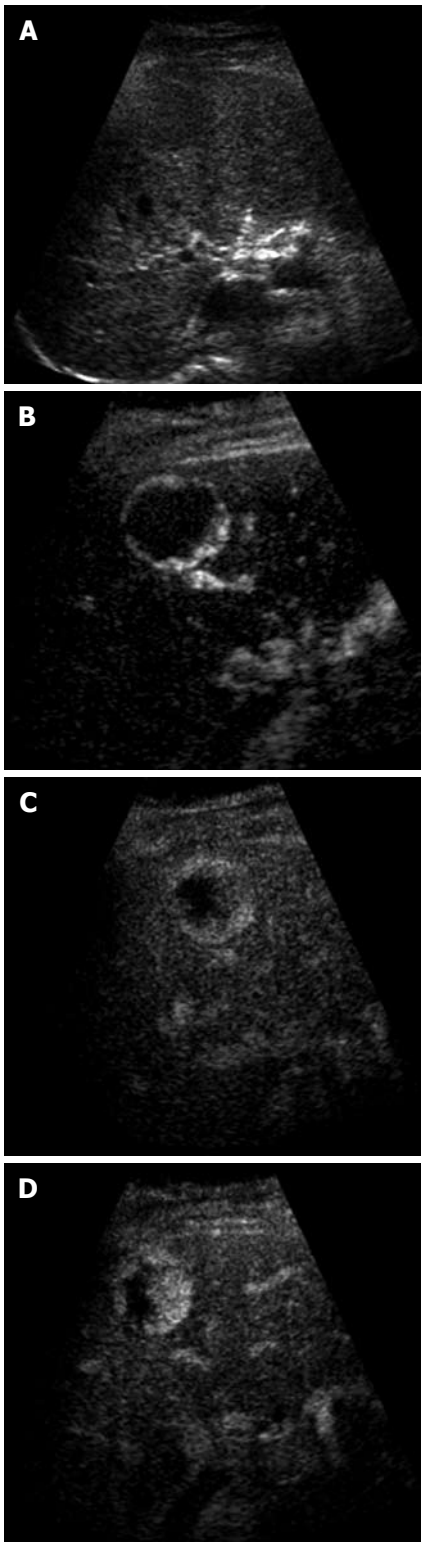

Figure 2 Haemangioma. A: Baseline focal hypo-echoic lesion; B: Arterial phase showing peripheral rim enhancement; C: Portal venous phase showing peripheral globular rim enhancement; D: Late phase showing progressive centripetal filling-in which is characteristic for hemangioma.

in assessing RFA ablation results in revealing earlier recurrences unsuspected clinically[29].

Assessment of tumour size and geometry

The selection of the RFA needle electrodes and the appropriate ablation protocols depend on the size of

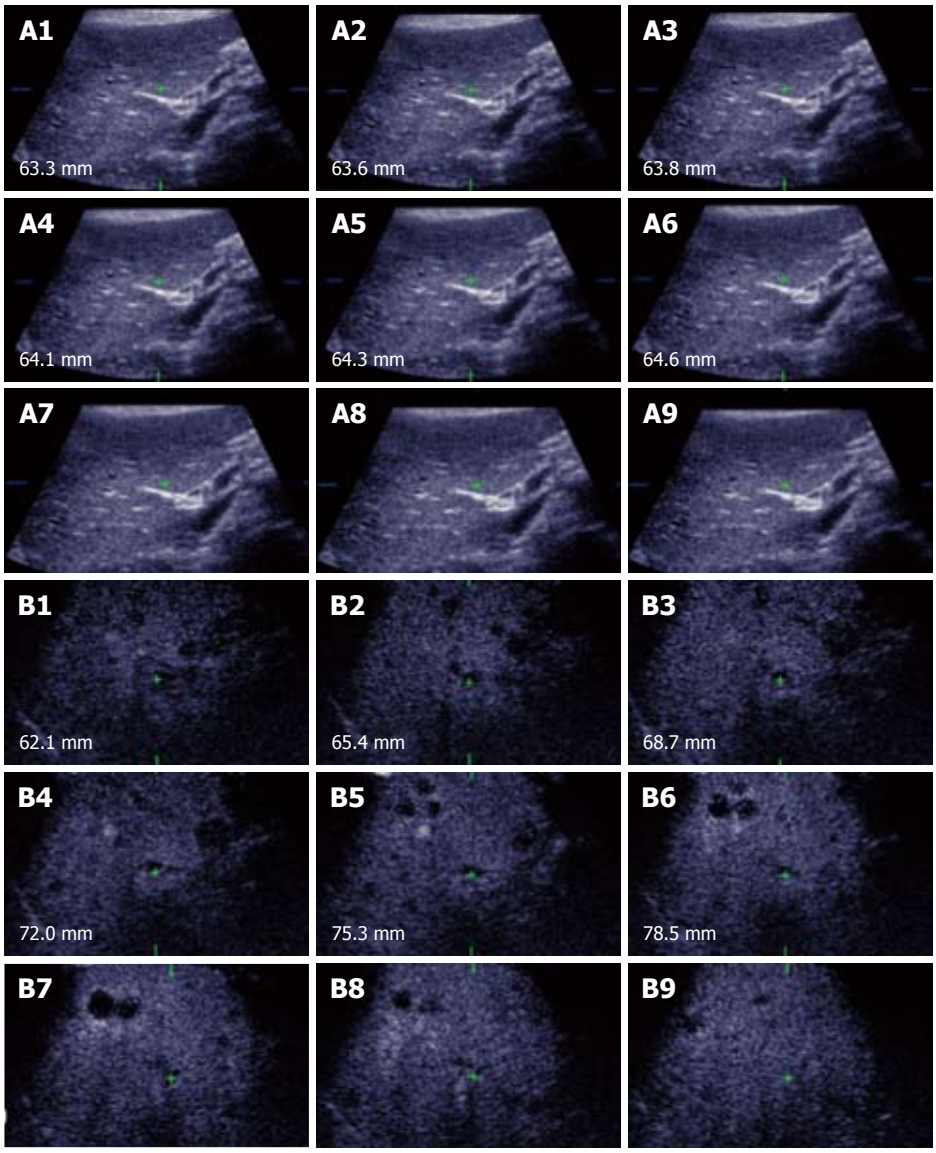

Figure 3 Unenhanced ultrasound and contrast enhanced 3D US. A: Unenhanced Fundamental mode ultrasound showing apparently normal liver; B: Contrast enhanced 3D US displayed as axial scans showing numerous occult metastases appearing as filling defects in the late phase.

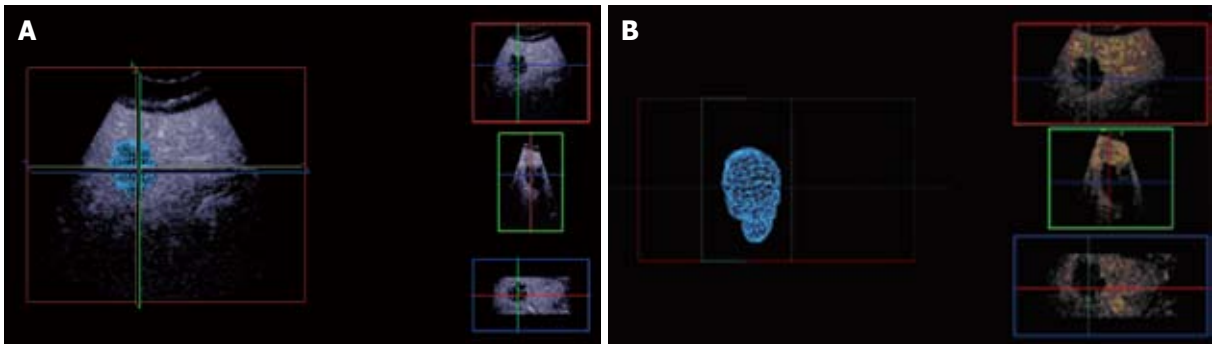

Figure 4 Multi-planar reconstruction. A: Tumor appears to be spherical in tumor modeling; B: Background liver subtraction shows geometry of the tumor model and long axis.

the tumor to be ablated. When the tumor is small (2 to 3 cm), its geometry is usually spherical. However, larger (> 3 cm) tumours may be elliptical. In locally advanced disease there may be aggregates of "daughter" hepatocellular carcinomas or "satellite" metastases merging as they grow resulting into lobular masses. Unenhanced and CE-3DUS assessment of the tumor geometry and determination of the lesional long axis is required to plan for the RFA targeting in order to restrict the number of RFA needle electrode insertions to the bare minimum to avoid seedling and potential complications (Figure 4). If the tumor is elongated, 3D assessment of the tumor geometry and its orientation relative to the probe position enables the planning of the insertion of the RFA needle electrode along the center of the tumor's long axis to treat the lower half of the

lesion first and subsequent withdrawal of the electrode to ablate the proximal residual tumor mass with a single puncture approach (Figure 4B).

Accurate delineation of the viable tumor margins in 3 orthogonal planes is a key in determining the true size of the lesion. Analogous to liver resection, ablation of the 0.5 to 1.0 cm layer of "normal" liver parenchyma around the tumor margin is important to limit subsequent local recurrence. Conventional US may be limited in delineating the true viable tumor margin; on the other hand CE-3DUS can accurately demonstrate the normal liver/tumor margin. Whilst this may not be critical for small lesions, with larger lesions a 5 mm error may significantly increase the risk of leaving residual disease. The importance of the true delineation and geometry is also largely related to the limited ablative capability of current RFA single or cluster needle electrodes which will produce at best a sphere of coagulation necrosis of 5 cm in diameter. Once the 3D data set has been acquired, the measurement of the tumor diameter in all 3 orthogonal planes determines the appropriate RFA needle electrodes.

Volume of the tumor can also be calculated automatically or manually depending on the software available. This can be done automatically following CE-3DUS using an "auto stacked contours" system, which automatically determines the tumor/normal liver border. First all 3 orthogonal planes are aligned into the center of the mass. The borders of the tumor are marked, and the number of slices for tracing the contours can be selected. Each contour is mapped and the tumor model created and its volume calculated automatically. Without the use of contrast, there is no automatic delineation of the tumor/normal liver border and the stacking of the tumor contours needs to be done manually which is more time consuming.

Targeting of tumors

Conventional ultrasound (2D) has been shown to be an excellent real-time tool in the placement of biopsy needles and RFA needle electrodes. Its limitation in the detection of occult liver tumors is well recognized compared with contrast enhanced CT and MR. Without the use of ultrasound contrast agents, these occult tumors could be localized using adjacent anatomical landmarks and then targeted blindly (i.e. without actual visualization of the tumor). CE-US facilitates the identification of these occult tumors for biopsy and/or for ablation. However, CE-US relies on the use of non-linear imaging mode to depict the contrast enhancement of the normal liver parenchyma with the malignant tumors appearing as filling defects in the late phase. Non-linear imaging mode is highly effective in subtracting native tissue linear echoes including those of the target biopsy needle or RFA needle electrodes. Whilst CE-US will identify the occult tumors, visualization of the biopsy needles and RFA needle electrodes is difficult on the non-linear imaging mode at low output power (Mechanical index). In the past, one had to switch between the non-linear imaging mode and fundamental modes screen to identify the position of the tumor and needle, respectively. However, with the advent of the side by side dual screen with the low MI fundamental mode and non-linear imaging mode simultaneously displaying the needle and the occult lesion, respectively, accurate real time targeting of occult lesions has been facilitated and significantly improves the work flow obviating the need to resort to CT or MRI scan guidance (Figure 5).

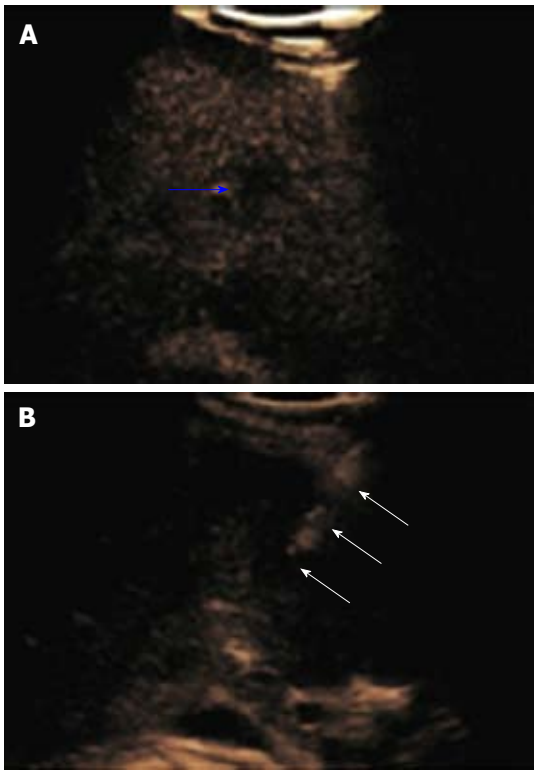

Figure 5 Side-by-side screen. Blue arrow points to small occult metastasis only seen on the CE-US non-linear imaging mode (A), whilst the open arrows point to the RFA needle electrode trajectory towards the occult metastasis, which is only visualized on the fundamental US scan (B).

With 2D US data acquisition, it is difficult to visualize mentally the 3D spatial aspects of the tumor and its specific relation to the surrounding hepatic vascular structures. CE-3DUS enables the modelling of the tumor geometry and determination of its long axis as well as its spatial relationship with the adjacent hepatic vascular anatomy and sometimes the extra-hepatic vital structures when the tumor is sub-capsular in location. The placement of the RFA needle electrodes can then be performed with reference to the probe position with the aid of a needle-guide or free-hand control (Figure 4). Furthermore, the added information provided by CE-3DUS also enables a more aggressive approach to the ablation of larger lesions beyond usage of a singular RFA needle electrode. Placement of multiple RFA needle electrodes will create coagulation necrosis even beyond the 7 cm (Figure 6). But the deployment of these multiple RFA needle electrodes needs to be accurate and is facilitated with CE-3DUS planning and guidance.

There are reports of improved needle localization

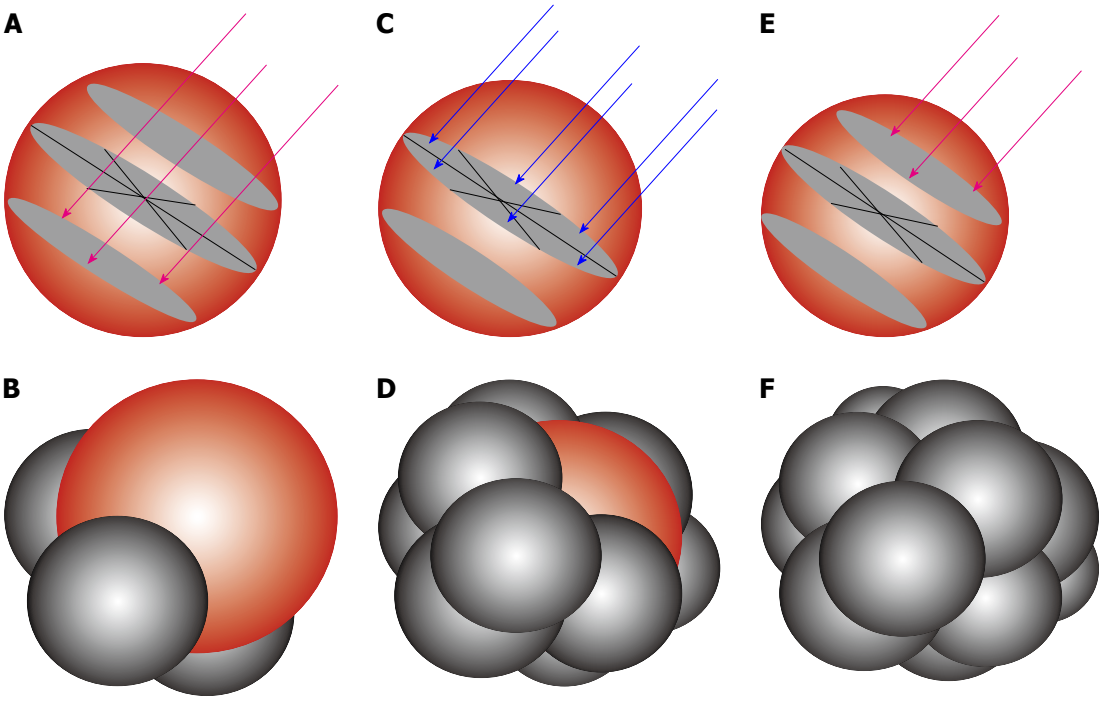

Figure 6 RFA needle electrode. Modeling to show the placement of the RFA needle electrodes to enable 12 overlapping spheres to give an equivalent of 7.5 cm diameter target sphere. (A, B) lower pole ablation with 3 RFA needle electrodes (red arrows) to create 3 overlapping ablation spheres (C, D) middle row ablation with 6 RFA needle electrodes (blue arrows) to create 6 overlapping ablation spheres and (E, F) upper pole ablation with retraction of the first 3 RFA electrodes to complete the target sphere of 7.5 cm.

Figure 7 RFA needle electrode with expandable antennas.

and guidance during biopsy using unenhanced 3D-US[30]. Earlier studies using unenhanced 3D-US to guide RFA needle electrodes have shown an increase in the confidence of the operator as well as a more accurate positioning of the electrodes in the majority of cases when compared with conventional 2D ultrasound guidance[31,32]. With regard to the accurate placement of RFA needle electrodes with expandable multiple antennas (Figure 7), particular attention needs to be observed (1) that the expandable antennas are deployed uniformly and symmetrically within the tumor to ensure margin (2) with lesions adjacent to vascular structures and sub-capsular location, that the latter are not punctured. 3D US is particularly valuable in the depiction of the safe deployment of these expandable antennas with the use of the elevation plane. 3D US enables the visualization of the RFA needle electrodes and the tumor simultaneously in the 3 orthogonal planes, among which the coronal and sagittal planes are aligned along the RFA needle electrode long axis whilst the elevation plane being perpendicular to it (Figures 8-10). Through translation or rotation within the acquired 3D volume, all regions of interest can be viewed from arbitrary orientations without any restriction of the transducer axis. The placement of multiple RFA needle electrodes can be planned following acquisition of the 3D volume data and accurate measurement of the distance between the electrodes and the tumor margin can be performed in advanced limiting the danger of unnecessary repeated electrode punctures through "trial and error" (Figure 11).

Monitoring of response

To assess the initial response to treatment, CE-3DUS can be performed only at 7 to 10 min after the end of RFA to allow for the dissipation of the native gas produced during the ablation process. Acquisition of 3D data set is carried out during the hepatic arterial, portal venous and late phase. Absence of any intra-lesional enhancement or moving microbubbles is consistent with complete coagulation necrosis and is easily depicted in hypervascular tumors. Residual viable tumor tissue is suspected when a portion of the original lesion maintains its micro-vascularity during the vascular phases. In enabling immediate further RFA of the residual disease, unnecessary delay may be avoided ensuring complete treatment within a single session. However, non-visualization of vascularity is not always a reliable indicator in the case of hypovascular

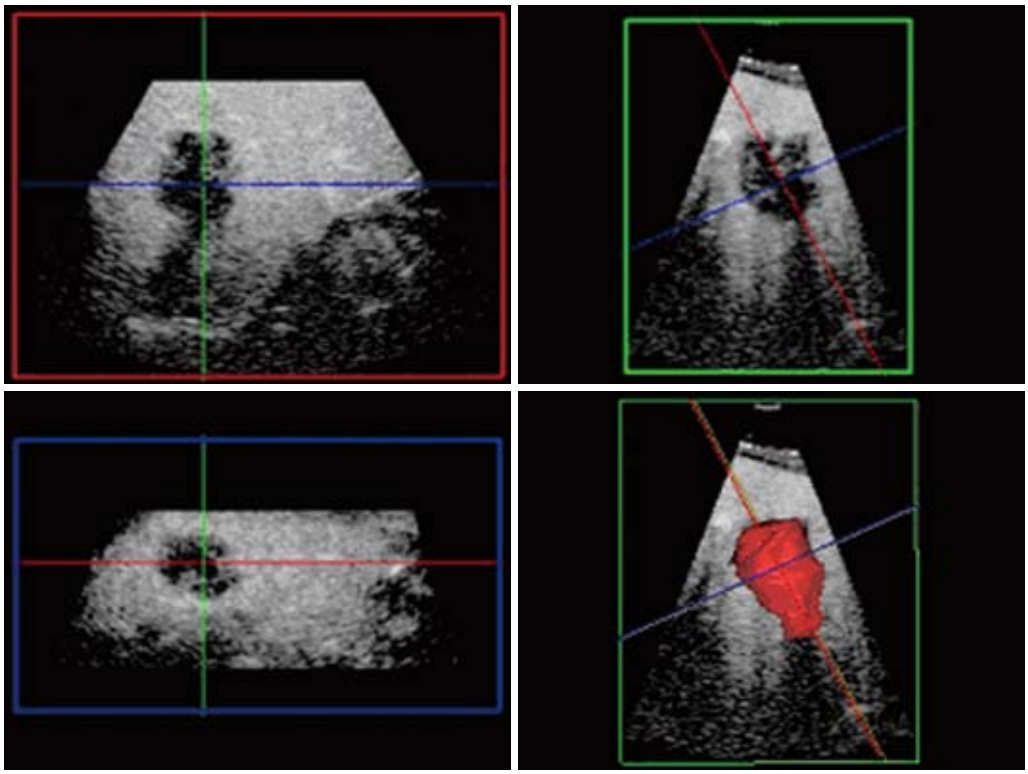

Figure 8 Multi-planar reconstruction. RFA Needle electrode placement along the long axis of the tumour mass which correspond to the planned "Red" axial plane bisecting the perpendicular the "Green" sagittal plane with reference to the probe position.

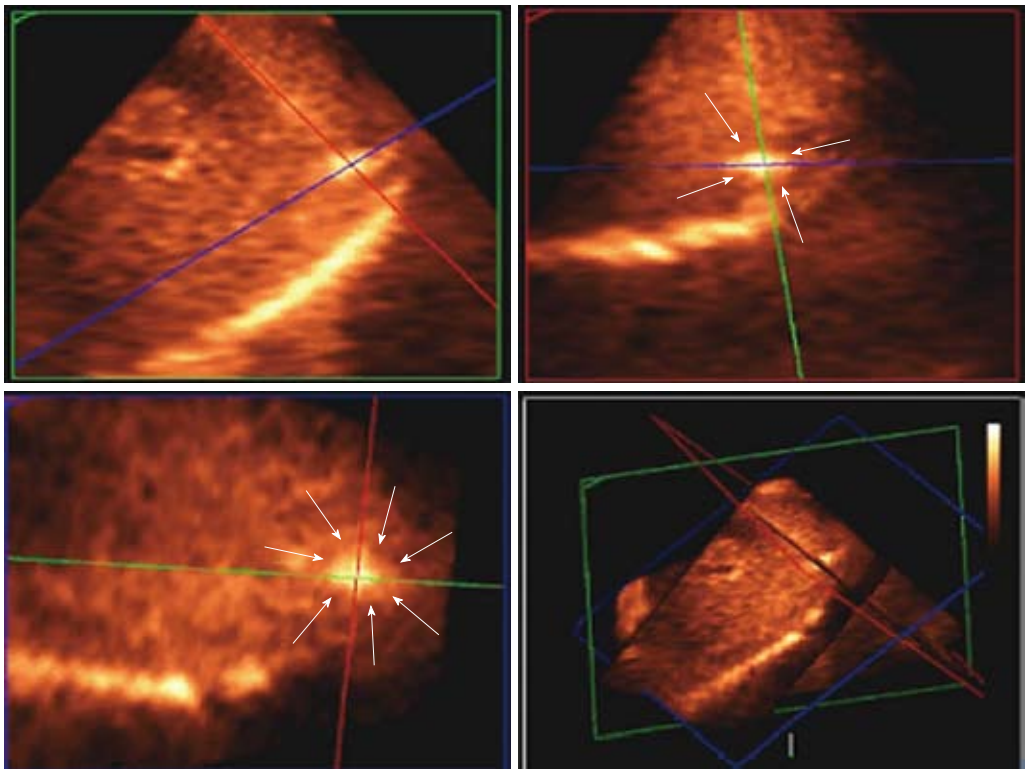

Figure 9 Matrix probe. 3D US showing the hyperechoic needle electrode tip and its spatial position in the tumour (white arrows delineates the tumour margin) in the subcapsular area and is clearly depicted in 3 orthogonal 2D images confirming the tip being in the center of the tumour.

tumors as in the case of most colorectal liver metastases. Complete coagulation necrosis may then be assessed through the side-by-side comparison of pre-RFA lesion size, volume and location, with those of the post-treatment coagulation necrosis. Peri-lesional "safety" margin adequacy must be evaluated at the same time. Clearly the advantage of CE-3DUS data acquisition during the vascular (arterial and portal) and late phases is evident to ascertain complete assessment of the volume of coagulation necrosis or presence of residual disease (Figure 12). Evaluation of the post RFA ablation volume relative to the pre RFA tumor volume may also be a particular additional important parameter in the assessment of response. A similar technique can also be used in the surveillance of these patients in the post ablative periods at 3, 6 or 12 mo; whilst this may not be optimal for patients with metastases who would benefit from CT in the surveillance for extra-hepatic disease,

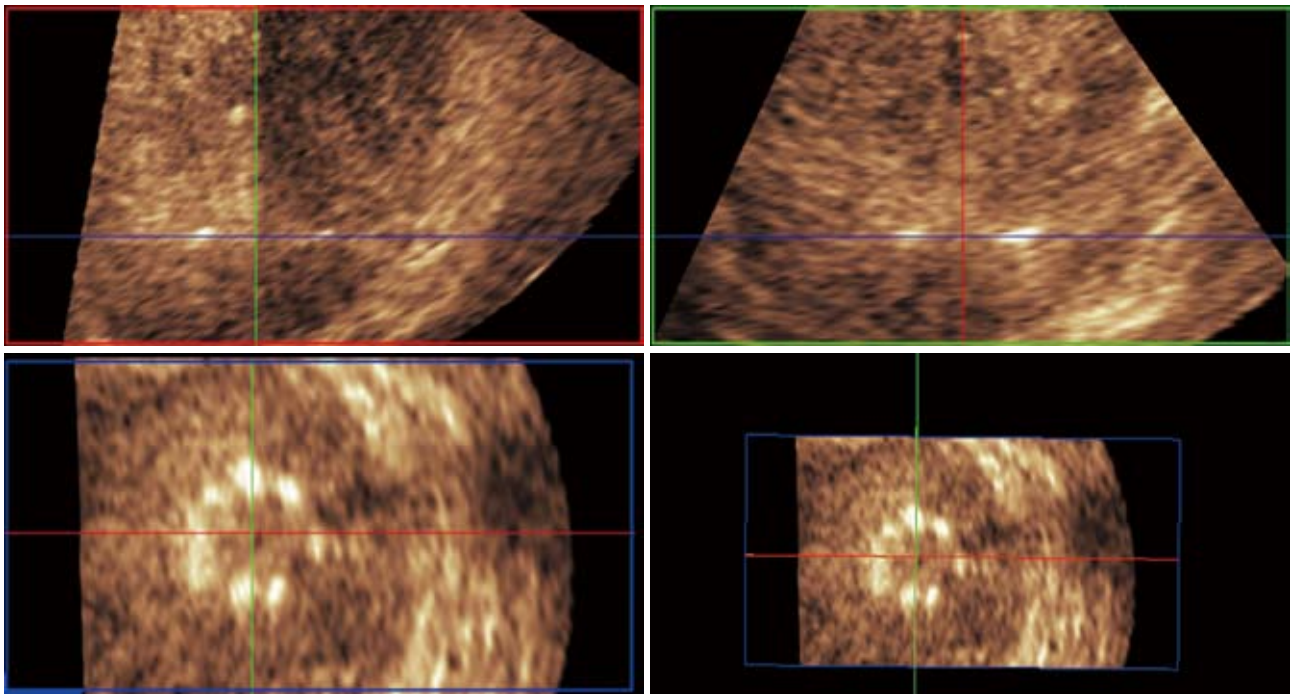

Figure 10 The 3 orthogonal planes showing the RFA needle electrodes with the multiple antennas deployed and clearly visualised on the (Blue: left lower quadrant) elevation plane following alignment of the Green and Red planes along the axis of the RFA needle electrode.

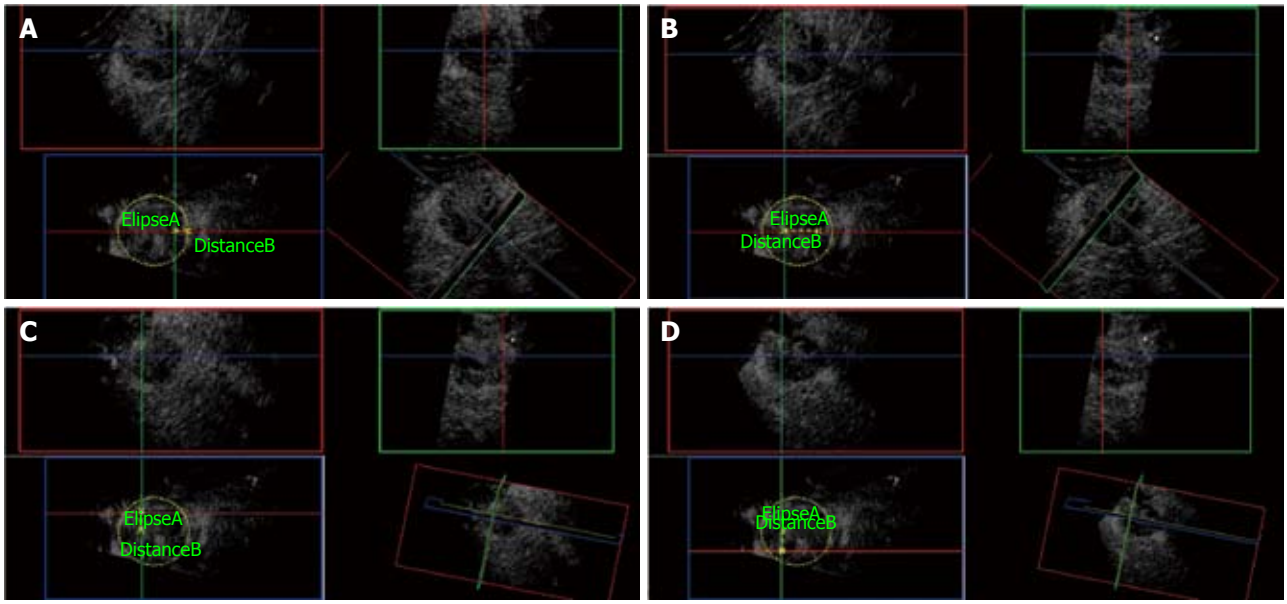

Figure 11 3D CE-US. A: Ellipse marks the tumor border at its maximal diameter (5 cm). First needle electrode insertion is planned from the elevation plane (Blue box) at 1 cm from the edge of the tumor. Green and Red orthogonal planes mark the trajectory of the needle electrode. B: Distance B measures 2.5 cm from the first needle electrode insertion and plans the plane (Green) of insertion for the next two needle electrodes. C: The second needle electrode is placed at the Green and Red planes intersection measuring 1.5 cm superior to the last position. D: Third needle insertion is placed at the Red and Green plane intersection, 3 cm below the second needle electrode as shown on the Blue plane.

it may be adequate for the surveillance of patients with hepatocellular carcinomas due to the much lower incidence of extra-hepatic dissemination.

Once local recurrence has been identified, CE-3DUS evaluation in the arterial phase and multi-planar reconstruction modelling are important in the planning for further RFA in that the geometry of the mass is usually non-spherical (Figure 13).

LIMITATIONS OF CE-3DUS

The spatial resolution of the current 3D probes is still limited on the non-linear imaging modes such that border delineation on the elevation plane is relatively poor. Volumetric acquisition of the data may be associated with distortion of the volume as a result of motion when using mechanical probes; however, this is not evident

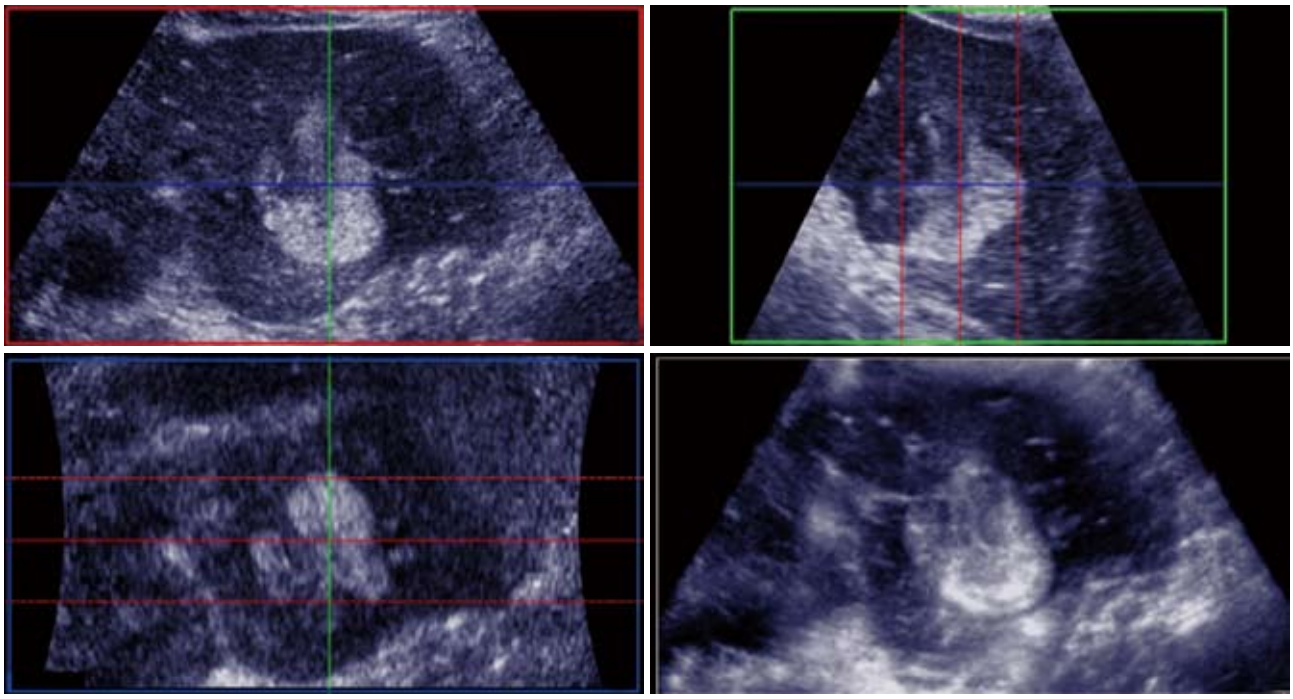

Figure 12 3D CE-US. Arterial phase 3D acquisition showing recurrent enhancing HCC in 3 orthogonal planes and the volume rendering analysis (left lower quadrant).

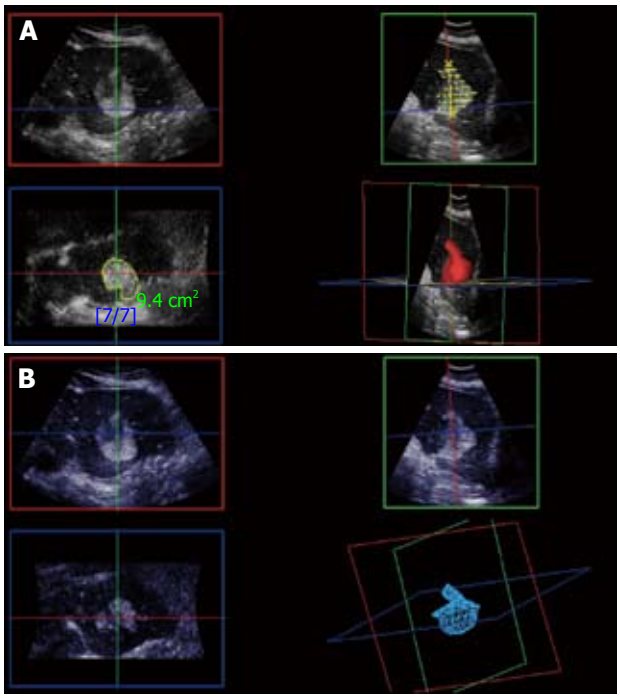

Figure 13 3D reconstruction of local recurrence geometry in the planning for further ablation. A: With normal liver background; B: Without normal liver background.

with the new electronic matrix probes largely due to the latter higher speed of volumetric scanning. The presence of native gas produced during the ablative process may produce shadowing artifacts, which may lead to inaccurate assessment of treatment response and calculation of ablation zone dimensions. There is a significant learning curve in the adoption of the new technique especially in the manipulation of the orthogonal planes.

FUTURE

One can expect further improvement in the spatial and temporal resolution of future 3D probes which would further facilitate live CE-3DUS targeting of the liver tumors in all three orthogonal planes simultaneously and enable real-time evaluation of the RFA needle electrodes alignment within the tumor volume on the elevation plane. Tissue perfusion is one of the functional parameters which can be used to assess tissue viability and may be used to assess response to treatment as well as a prognostic indicator. Whilst tissue perfusion quantification can be assessed on CE 2D-US, it is only valid if there is absolutely no movement during the data acquisition. Perfusion assessment along one single plane scan is also not representative of the whole tumor mass perfusion. Clearly 3D perfusion quantification is urgently needed for accurate functional imaging which may be incorporated into routine clinical practice through parametric imaging.

CONCLUSION

CE-3DUS is useful in the radiofrequency ablation (RFA) of liver tumors with improved detection and display of occult lesions and recurrence, in the assessment of lesional geometry and orientation for a more accurate planning and guidance of multiple RFA needles electrodes in large tumors and in the evaluation of residual or recurrent disease within the immediate and/or subsequent follow-up periods.

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ORIGINAL ARTICLES

Transient and etiology-related transcription regulation in cirrhosis prior to hepatocellular carcinoma occurrence

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Abstract

AIM: To search for transcription dysregulation that could (1) differentiate hepatocellular carcinoma (HCC)-free from HCC-related cirrhosis (2) differentiate HCC-free cirrhosis related to HCV from that related to alcohol intake.

METHODS: Using microarray analysis, we compared transcript levels in HCC-free cirrhosis (alcoholism: 7; hepatitis C: 7), HCC-associated cirrhosis (alcoholism: 10; hepatitis C: 10) and eight control livers. The identified transcripts were validated by qRT-PCR in an independent cohort of 45 samples (20 HCC-free cirrhosis; 15 HCC-associated cirrhosis and 10 control livers). We also confirmed our results by immunohistochemistry.

RESULTS: In HCC-free livers, we identified 70 transcripts which differentiated between alcoholic-related cirrhosis, HCV-related cirrhosis and control livers. They mainly corresponded to down-regulation. Dysregulation of Signal Transduction and Activator of Transcription-3 (STAT-3) was found along with related changes in STAT-3 targets which occurred in an etiology-dependent fashion in HCC-free cirrhosis. In contrast, in HCC, such transcription dysregulations were not observed.

CONCLUSION: We report that transcriptional dysregulations exist in HCC-free cirrhosis, are transiently observed prior to detectable HCC onset and may be appear like markers from cirrhosis to HCC transition.

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Key words: Liver; Pathology; Alcoholism; Hepatitis C virus; Gene expression; Carcinogenesis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most prominent, primary liver cancer. Its main etiologies are viral hepatitis B or C (HBV; HCV), alcoholism or aflatoxin B1 intoxication, with both HCV and alcoholism currently increasing in incidence in Western countries and predominating as etiologies[1,2]. In most instances, HCC develops in the setting of chronic hepatitis and/or cirrhosis[3]. Cirrhosis is the end stage of a chronic liver disease which results in regenerating nodules surrounding by fibrous septa and, ultimately, may lead to cancerous nodules. HCC has a poor prognosis but,

apart from surgery, no major improvements in disease therapies have been recently reported[4], most likely because the heterogeneity of the disease and its various etiologies prevent any progress in our understanding of HCC development and mechanisms[5].

Numerous genome-wide analyses of abnormal gene expression in HCC as compared to normal, control livers, have resulted in identification of gene sets with altered expression[2,6,7], part of which result from underlying gene mutations and/or chromosome alterations[8-10] and account for a limited number of altered pathways[9,11]. A few similar studies have been done in HCC-free cirrhosis and they mostly considered markers for a pre-HCC condition[12], or selected pathways[13], or mixed etiologies[12,14]. In fact, the number of comparative studies devoted to HCC etiology has remained scarce, whether this was done in a clinical setting[15-19], cell lines[20] or animal models with oncogene overexpression[21]. In particular, the viral etiologies have been considered[17,18,20,22] whereas abnormal gene expression in alcoholism-dependent HCC has received very little attention. Therefore, the impact of etiology still remains an important issue[7,23]. We recently reported that a number of genome-wide abnormalities in alcoholism-associated *vs* HCV-associated HCC are etiology-dependent and some of them are of pathological relevance[24]. Remarkably, the abnormal transcription levels that differentiate HCC nodules in an alcoholism-dependent *vs* HCV-dependent fashion can no longer discriminate between both etiologies when transcripts are measured in the surrounding cirrhosis[24]. Yet, any etiology-dependent abnormalities that could be observed in HCC-free cirrhosis would be of interest. We investigated whether some transcription dysregulations could be found in HCC-free cirrhosis in an etiology-dependent fashion. Furthermore, we searched and found transcript dysregulations that differentiate HCC-free cirrhosis from peritumoral cirrhosis. We now report that such transcription dysregulations do exist in HCC-free cirrhosis and are observed prior to detectable HCC onset.

MATERIALS AND METHODS

Human subjects and tissue sampling

Non-alcoholic steatohepatitis, primary biliary cirrhosis and infant biliary atresia were excluded from this study. Chronic alcohol abuse was estimated as detailed[24]. HBV and HCV infections were serologically determined in every patient and any HBV-positive patient was excluded. Patients with an HCC-free or HCC-associated cirrhosis were histologically diagnosed by trained pathologists (AF, PBS). Liver fragments came to our laboratory from the digestive surgery unit of Charles Nicolle Hospital (Rouen, France) or the pathology unit of Pellegrin Hospital (Bordeaux, France) under strict anonymity. HCC-free, cirrhotic tissue was obtained from transplanted patients. Peri-tumoral, cirrhotic tissue was taken at a distance from HCC resection whenever the latter was excised for curative purposes. Control, non-cirrhotic human liver (CL) was obtained from patients operated on for a benign liver tumor or metastasis of a non-hepatic cancer. According to the current French rules and ethical guidelines, neither informed consent nor advice from an ethical committee were requested prior to RNA analysis in tissues that would otherwise be disposed of. Various clinical features in a total of 69 cirrhosis without HCC, (n = 34) or with HCC (n = 35), as well as in a set of 18 histologically normal CLs are summarized in Table 1. A METAVIR score from the combined extents of inflammation (A0-A3) and fibrosis (F0-F4) were histologically diagnosed by trained pathologists (AF, PBS).

Transcriptome analysis and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA extraction from tissues stored at -80℃ was done with Trizol. Our set of human cDNA probes dubbed *Liverpool* and tailored to a complete coverage of the human liver transcriptome under healthy or pathological conditions (ca. 10^4 genes), the associated *LiverTools* database, as well as the procedures from array preparation to data handling have all been detailed[25]. In brief, every RNA sample was subjected to three rounds of hybridization and the resulting signals were normalized from the average signal of every spot (mean grey) on the matching hybridization image. The mean signal per transcript was used for selections of significantly regulated transcripts. Probe re-sequencing was done with an ABI3100 capillary sequencer (Applied Biosystems, Foster City, USA). Real-time qRT-PCRs of transcripts were done with a Light Cycler (Roche Diagnostics, Manheim, Germany). Transcript normalization was done with the 18S RNA. The primers designed with the Primer3 software (http://frodo.wi.mit.edu) are listed in a Table 2.

Data mining

Our raw data are deposited in the GEO repository under accession number GSE10356. The TIGR Multiexperiment viewer (Tmev version 2.2, http://www.tm4.org) was used for (1) unsupervised hierarchical clustering (UHC) using the Manhattan distance and complete linkage options; (2) supervised analyses such as the *t*-test or ANOVA adjusted with Bonferroni's correction or K-nearest neighbour classification (KNNC) and (3) evaluation of sample re-assignment by a random procedure (jackknife-10^6 iterations). Another, supervised classification was done by Support Vector Machine (SVM) (http://svm.sdsc.edu/). The Gene Ontology Tree Machine (GOTM) program (http://bioinfo.vanderbilt.edu/gotm/) was used to categorize protein function(s) by ontology. Detailed protein functions were retrieved with the SOURCE (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch) and/or OMIM (http://www.ncbi.nlm.nih.gov/sites/entrez) tools. Protein networks were identified with Bibliosphere (www.genomatix.de). Statistics were carried out with the GraphPad Instat software, version 3 (http://www.graphpad.com/).

Table 1 Biological and clinical data from patients with cirrhosis alone, HCC-associated cirrhosis and controls

Patient[1]	Number	Male/Female	Age[2]	Pathology[3]	Etiology[4]	Metavir[5]			
						A0	A1	A2	A3
A1 to A7	7	3/4	48.4 ± 3.2	CIR	ALC	0	4	1	2
A15 to A24	10	7/3	50.4 ± 7.5	CIR	ALC	0	8	2	0
PA1 to PA10	10	8/2	67.1 ± 8.0	CIR + HCC	ALC	2	5	3	0
PA21 to PA29	9	9/0	60.9 ± 8.2	CIR + HCC	ALC	1	7	1	0
V8 to V14	7	6/1	57.4 ± 8.9	CIR	HCV	0	2	4	1
V25 to V34	10	5/5	55.7 ± 16.0	CIR	HCV	0	5	3	2
PV11 to PV20	10	6/4	71.5 ± 5.1	CIR + HCC	HCV	0	1	6	3
PV30 to PV35	6	2/4	66.2 ± 9.5	CIR + HCC	HCV	0	1	3	2
CL1 to CL8	8	3/5	59.9 ± 15.9	No CIR, no HCC[6]	--	7	1	0	0
CL9 to CL18	10	2/8	49.5 ± 14.0	No CIR, no HCC[6]	--	9	1	0	0

[1]HCC-free alcoholic cirrhosis; PA: Peritumoral alcoholic cirrhosis; V: HCC-free HCV cirrhosis; PV: Peritumoral HCV cirrhosis; CL: Control without any detectable fibrosis. Underlined samples were studied by microarray and qRT-PCR; No underlining, independent cohort of samples studied by qRT-PCR only. [2]mean ± SD. [3]CIR: Cirrhosis; CIR + HCC: Peritumoral cirrhosis. [4]HCV: Hepatitis C virus infection; ALC: Alcoholism; --: None. [5]From left to right, number of patients with a given score of inflammation A0-A3. Difference in METAVIR score in HCC-free cirrhosis between all V *vs* A patients, $P = 0.17$ (Mann and Whitney's test); In peritumoral cirrhosis between all PV *vs* PA patients, $P = 0.005$. [6]Histologically normal liver sampled at a distance from a benign liver tumor or from a metastasis of non-hepatic cancer.

Table 2 Oligonucleotides for qRT-PCR

Oligonucleotides	Forward	Reverse	Amplicon size (bp)
ACSM2	AAATCCCGACAAGACAGCAG	CTGATCACAGCCGTCTCAAC	201
GSTA1/2	TCTGCAGAAGATTTGGACAAG	TCAATCAGGGCTCTCTCCTT	170
ADH4	GTCTGCTTGGATGTGGGTTT	TGATTCTGGAAGCTCCTGCT	150
HSCARG	GAAACTGGTGGTGGTTTTCG	CATCTTGGTCTCCCTGCACT	170
AHNAK	CAAAGGGAAACACACCGACT	GCTCTCAGCAGTCAATGCAA	207
HSD17B6	TCTGGGGACTGGTGAACAAT	GTGCTCTCCTCACCAAAGGA	150
APOH	CCGAGGAGGGATGAGAAAGT	AGAATCAGCGCCATTCAGAT	193
IFI27	CCAAGCTTAAGACGGTGAGG	AAAACTACGGCAGAGCCAGA	196
ARID1A	CTACGCTGCCACGTGTGTAT	GTACAGCATCGCACCAAGAG	187
MT1G	TCCTGCAAGTGCAAAGAGTG	ACTTCTCCGATGCCCCTTT	118
ARL2BP	AGGATGAAGTGGCTGGTGAC	GGAAGCTGGCAGAGAAGATG	170
ORM2	TTATATCGCATCGGCCTTTC	CCGCTGGACATTCAGGTAAC	172
ATP5G2	TTGTCTCCACTCCCTCCTTG	TGTGTCGATGTCCCTTGAAA	191
PLG	GTAGGTGGTCCCTGGTGCTA	CCTACAACCCTTCCAGGACA	137
CYP3A4	CTTTGGAAGTGGACCCAGAA	CGGGTTTTTCTGGTTGAAGA	164
STAT3	CCCCATACCTGAAGACCAAG	CTCCGAGGTCAACTCCATGT	185
CYP2E1	TCAAGCCATTTTCCACAGGA	CGATATCCTTTGGGTCAACGA	129
TIMP1	AATTCCGACCTCGTCATCAG	GTTGTGGGACCTGTGGAAGT	195
DPF2	CTCCTGGCTCACTCTTACGG	AAGGGGATTTTGGAGGTAGG	211
18S	GTGGAGCGATTTGTCTGGTT	CGCTGAGCCAGTCAGTGTAG	200
DPM1	GCAGTCCACGACAGAACAAA	CATCTGGGCTTCCATCATCT	150

Immunohistochemistry

D4 zinc and double PHD fingers family 2 (DPF2) and plasminogen (PLG) protein levels were assessed in formaldehyde-fixed, paraffin-embedded, 5 mm thick liver section samples giving a total of 40 other cirrhosis without or with HCC (10 alcoholic and 10 HCV in each group), as well as a set of 10 other histologically normal CLs. This assessment was effected by immunohistochemistry using the ultraView™ Universal DAB Detection Kit following the manufacturers instructions (Ventana Medical systems) with mouse anti-DPF2 IgGs (Abnova corporation) at 1 μg/mL or mouse anti-PLG IgGs (Interchim) at 38 μg/mL.

The percentage of positive cells was evaluated on the whole surface of the histological section and the staining intensity was estimated. Two scores from 0 to 4 were given as two independent visual scores by a trained pathologist and were evaluated using a LEICA DMR microscope equipped with a camera. The number of positive cells or P score was: 0, no positivity; 1, < 25%; 2, 25%-50%; 3, 50%-75% and 4, > 75%-100%. The determination of immunostaining intensity or I score was: 0, no staining; 1, very weak staining only seen at magnification × 10; 2, staining obviously seen at magnification × 10; 3, moderate staining seen at magnification × 2.5; 4, strong staining seen at magnification × 2.5. The IP score was obtained from the additional combination of the two parameters I + P.

RESULTS

Different transcriptome alterations in HCC-free or peritumoral cirrhosis vs CLs

First, with microarray data from 14 HCC-free cirrhosis samples (A1-A7, V8-V14) and eight CLs (CL1-CL8), we identified 30 transcripts

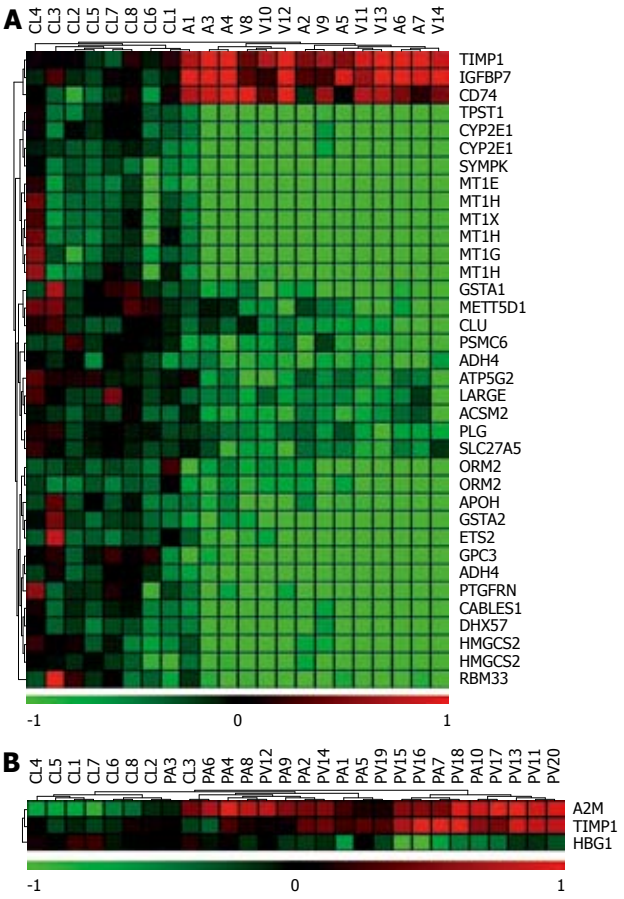

Figure 1 Clustering of cirrhosis from comparisons of transcript levels *vs* CIs. Every transcript level expressed as a [level per patient/median level in CLs] was measured by microarray. The samples are shown as a dendrogram on top and the transcripts are listed vertically. Bottom scale bar (log2 scale): decreased (green), increased (red) or unchanged (black) transcript level. A: UHC was made in 14 HCC-free cirrhosis samples and 8 CLs, from 30 transcript levels first identified as cirrhosis markers in an HCC-free context. B: UHC was made with 20 peritumoral cirrhosis samples and 8 CLs, from 3 transcript levels identified as cirrhosis markers in an HCC context.

whose levels differed between [A + V] cirrhosis *vs* CLs (*t*-test adjusted by Bonferroni's correction, $P < 0.05$, Figure 1A). In contrast, similar data from 20 peritumoral cirrhosis samples (PA1-PA10, PV11-PV20) and eight CLs identified only three transcripts, but they did not distinguish [PA-PV] from CLs (*t*-test adjusted by Bonferroni's correction, $P < 0.05$, Figure 1B). This HCC-dependent difference in transcript number was significant (30 *vs* 3, Fisher's test, $P < 0.0001$). Furthermore, the expression levels of the 30 transcripts, which distinguished HCC-free cirrhosis, from CLs, did not distinguish [PA-PV] from CLs (data not shown). Thus, we identified transcripts which were dysregulated in HCC-free cirrhosis but not in peritumoral cirrhosis.

Different transcriptome alterations in alcoholic- vs HCV cirrhosis vs CLs

Next, the comparison of transcript levels in alcoholic HCC-free cirrhosis *vs* CLs identified 10 dysregulated transcripts (*t*-test adjusted by Bonferroni's correction, $P < 0.05$). Likewise, we identified 49 dysregulated transcripts in HCV HCC-free cirrhosis *vs* CLs. We also found 33 transcripts that were differentially expressed when directly comparing alcoholic *vs* HCV HCC-free cirrhosis. Overall, we obtained a non-redundant list of 70 transcripts whose levels were able to completely distinguish between the three groups by UHC: alcoholic, HCV HCC-free cirrhosis and CLs (Figure 2A). This was supported by a jackknife procedure (100% success). In contrast, in HCC five transcripts failed to properly distinguish between these three groups (*t*-test adjusted by Bonferroni's correction, $P < 0.05$, Figure 2B). This HCC-related difference was significant (70 *vs* 5, Fisher's test, $P < 0.0001$). Furthermore, the expression levels of these 70 transcripts, which separated alcoholic, HCV and CLs in an HCC-free context, did not distinguish between them in an HCC context (data not shown).

Table 3 Performance of various, unsupervised or supervised classification tools for HCC-free cirrhosis samples

Samples[2]	Tool[1]		
	UHC (%)	SVM (%)	KNNC (%)
A1-A7 + A15-A24	65[3]	100	100
V8-V14 + V25-V34	71	80	70
CL1-CL18	100	70	80
All test samples	79	83	83

[1]Unsupervised hierarchical clustering (UHC) was done with 52 cirrhotic or CL samples. Supervised training/testing procedures (KNNC; SVM) were each done by first separating these 52 samples into 22 training (A1-A7, V8-V14 and CL1-CL8) and 30 test samples (A15-A24, V25-V34 and CL9-CL18). [2]20 transcript levels were measured in total RNA from every sample by qRT-PCR. These transcripts had the most significant difference in levels (Bonferroni-corrected ANOVA, $P < 0.05$) between alcoholism *vs* HCV *vs* CLs (ACSM2, ADH4, AHNAK, APOH, ARID1A, ARL2BP, ATP5G2, CYP2E1, CYP3A4, DPF2, DPM1, GSTA 1-2, HSCARG, HSD17B6, IFI27, MT1G, ORM2, PLG, STAT3, TIMP1). [3]% of properly classified test samples.

From our list of 70 transcripts obtained from our microarray data, we measured by real-time qRT-PCR the 20 most discriminant transcripts (as listed in Table 3, footnote 2) which were differentially expressed according to etiology. Using both the 22 training samples (A1-A7, V8-V14 and CL1-CL8) and 30 further independent test samples (A15-A24, V25-V34 and CL9-CL18) in order to classify HCC-free cirrhosis by unsupervised (UHC) or supervised training/testing procedures (KNNC and SVM),we found that these 20 transcripts resulted in a classification accuracy of 79%-83% test samples (Table 3).

Most transcriptome alterations in HCC-free cirrhosis are a transient event

The abnormalities in transcript levels seen in HCC-free cirrhosis, but not in peritumoral cirrhosis, were further evaluated timewise. As shown in Figure 3A (upper left star) the expression levels in CLs ($n = 18$), HCC-free cirrhosis ($n = 34$) and peritumoral cirrhosis ($n = 35$) were measured by qRT-PCR. When comparing the above 20 transcript levels between HCC-free *vs* CLs, their mean level in HCC-free cirrhosis was up-regulated (TIMP1), unchanged (3/20 transcripts, 15%, DPF2, IFI27, STAT3),

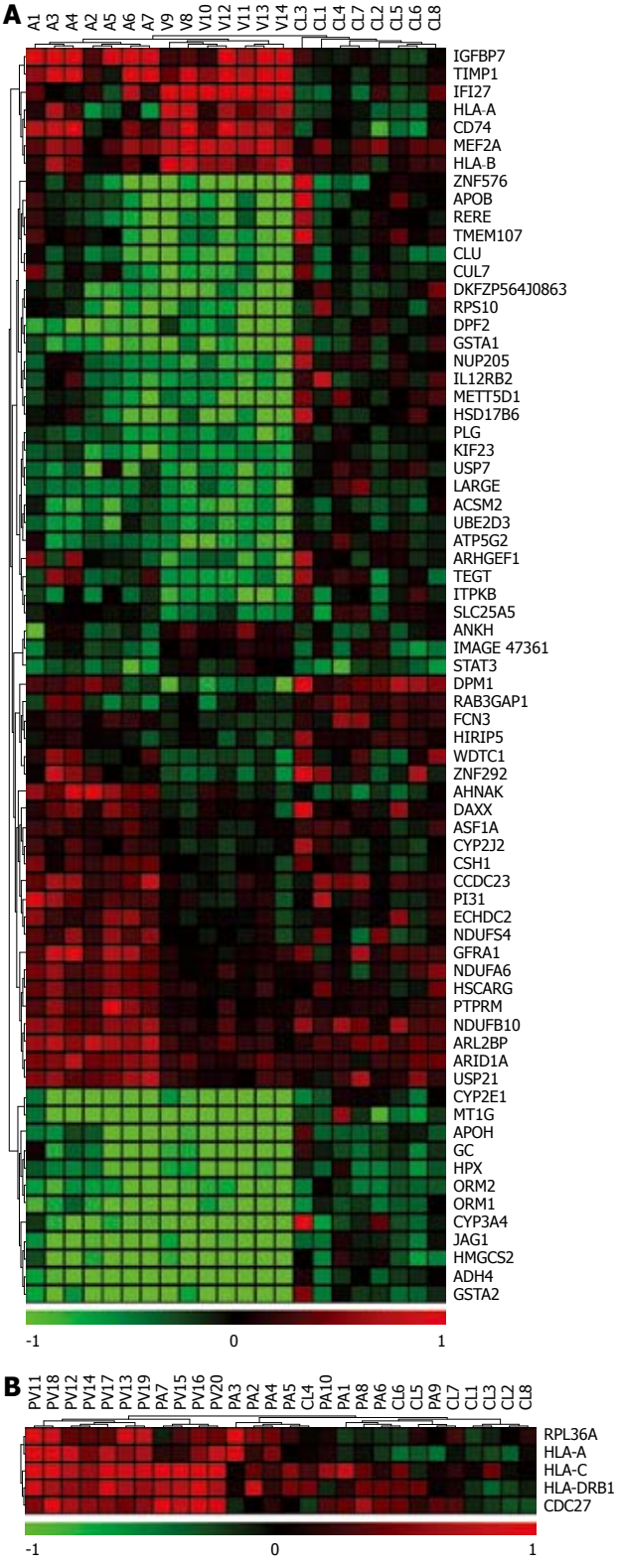

Figure 2 Clustering of cirrhosis samples from transcript levels. Every transcript was measured by microarray and expressed as a [level per patient/median level in CLs]. The samples are shown as a dendrogram on top and the transcripts are listed vertically. Bottom scale bar (log2 scale): decreased (green), increased (red) or unchanged (black) transcript level. A: UHC was made in 14 HCC-free cirrhosis samples and eight CLs, from 70 altered transcript levels first identified as markers of HCC-free cirrhosis. A or V, alcoholism-related or HCV-related etiology B: UHC was made in 20 HCC-associated cirrhosis samples and eight CLs, from five transcript levels identified by *t*-test adjusted by Bonferroni's correction. PA or PV, perinodular cirrhosis, with an alcoholism-related or HCV-related etiology, respectively.

or mostly down-regulated (16/20, 80%). In contrast, this down-regulation was not found when cirrhosis was associated with HCC. Indeed, in peri-tumoral cirrhosis a significant return to the CL level or even an up-regulation was observed (Figure 3A, upper right star). Moreover, as shown in Figure 3B, 9/20 (45%) transcript levels further displayed etiology-dependent differences found (1) only in HCC-free cirrhosis (alcoholism *vs* HCV, lower left star, 6/9 transcripts, 66%), or (2) only in peritumoral cirrhosis (lower right star, 2/9 transcripts, 22%, ARID1A, ORM2), or (3) in both (IFI27). Overall, these transcript dysregulations were mostly seen in HCC-free cirrhosis, often resulted from a transient down-regulation, and half of them were etiology-dependent in agreement with the initial selection.

Semi-quantitative immunodetection of DPF2 and PLG in liver samples

Among the nine genes mentioned above which displayed etiology-dependent differences, DPF2 and PLG, whose antibodies were marketed for immunohistochemistry use, were selected. We quantified their protein levels in a total of 40 other cirrhosis without or with HCC (10 alcoholic and 10 HCV in each group), as well as in a set of 10 other histologically normal CLs.

The DPF2 protein level was significantly higher in HCC-free cirrhosis, and then decreased in HCC-associated cirrhosis to return to a level similar to that observed in CLs, but this level regulation was mild (data not shown).

The PLG protein level was also significantly different in CLs, HCC-free and HCC-associated cirrhosis. Indeed, the PLG level was significantly lower in HCC-free cirrhosis as compared to that observed in CLs and then increased in HCC-associated cirrhosis to return to a level quite similar to that observed in CLs (Figure 4A). The immunohistochemical pattern for CLs with a strong hepatocellular staining was shown in Figure 4D). The PLG protein level also displayed etiology-dependent differences. Indeed, the decrease of the staining was significantly higher in HCC-free HCV cirrhosis (Figure 4E) than in HCC-free alcoholic cirrhosis (Figure 4B) and in HCC-associated cirrhosis the increase with a return to the baseline was the same whatever the etiology (HCV or alcohol) (Figure 4C and F). Thus, the PLG protein level confirmed our results obtained at the transcriptional level.

Functional differences in HCC-free vs peritumoral cirrhosis

We first investigated whether transcript variations could point to functional dysregulation in HCC-free cirrhosis. By comparing our list of 70 transcripts and our *Liverpool*, a different frequency of dysregulated transcripts in eight functional subsets was found (detailed as a Table 4): (1) cell proliferation ($P = 0.01$); (2) regulation of cell migration ($P = 0.009$); (3) blood vessel development ($P = 0.007$); (4) lipid metabolism ($P = 0.007$); (5) antigen processing and presentation

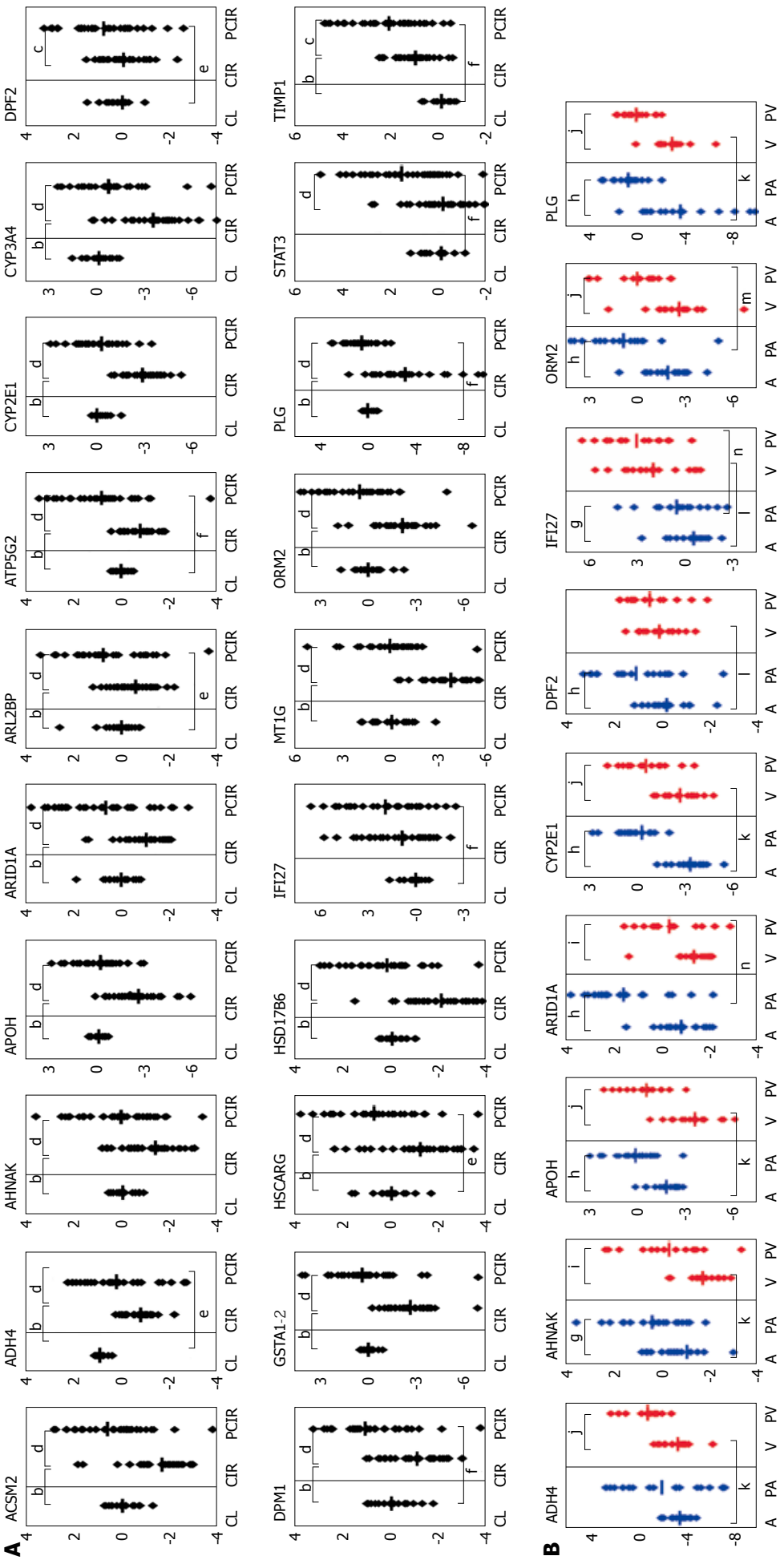

Figure 3 Changes in transcript levels in cirrhosis. The transcripts are those listed in Table 3, footnote 2F. Their levels were determined by real-time qRT-PCR in training and testing samples (18 CLs, 34 HCC-free cirrhosis and 35 peritumoral cirrhosis). Every transcript name is noted on top and its level per cirrhosis type is expressed on the ordinate as a log2 [level in type/median level in CLs]. The mean value of transcripts is shown as an horizontal bar. A : CL: control; CIR: HCC-free cirrhosis (regardless of etiology); PCIR: perinodular cirrhosis (regardless of etiology). Significant difference between CIR and CL ($^{a}P < 0.05$, $^{b}P < 0.01$, Mann-Whitney *U* test), between CIR and PCIR ($^{c}P < 0.05$, $^{d}P < 0.01$) and between PCIR and CL ($^{e}P < 0.05$, $^{f}P < 0.01$). B: Transcripts separated per etiology : alcoholism (blue), HCV (red); A or V: alcoholic or viral, HCC-free cirrhosis; PA or PV: perinodular, alcoholic or viral cirrhosis. Significant difference between A and PA ($^{g}P < 0.05$, $^{h}P < 0.01$), between V and PV ($^{i}P < 0.05$, $^{j}P < 0.01$), between A and V ($^{k}P < 0.05$, $^{l}P < 0.01$) and between PA and PV ($^{m}P < 0.05$, $^{n}P < 0.01$).

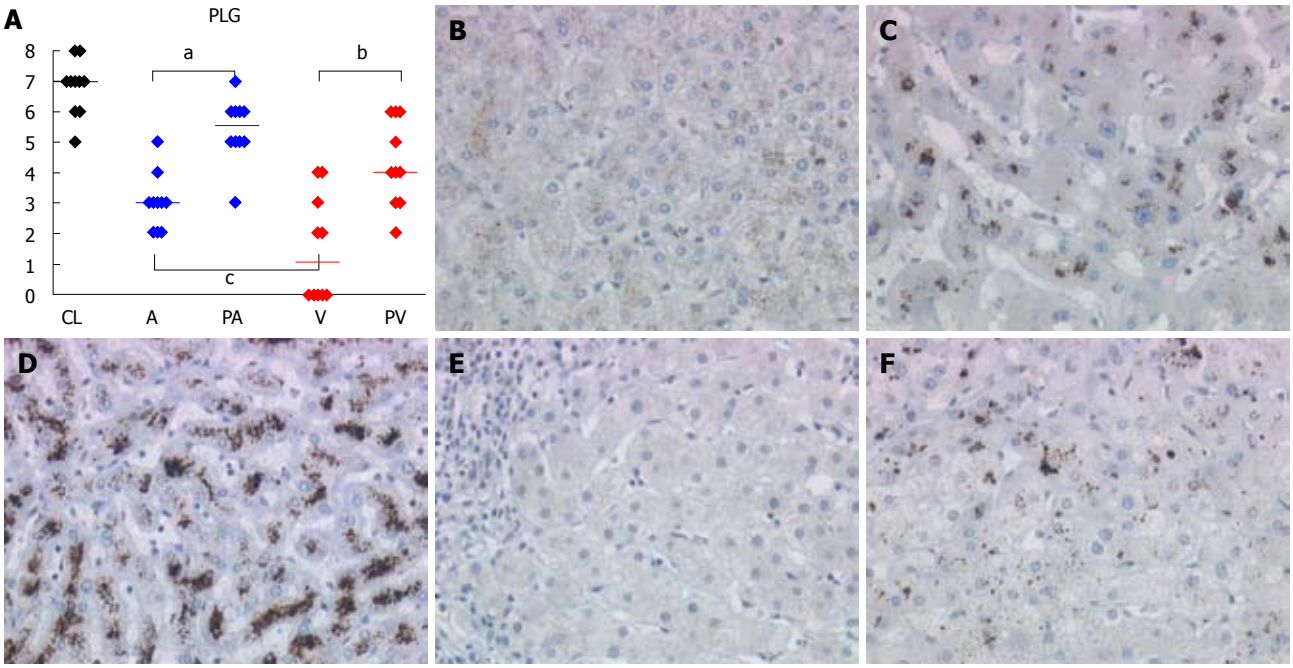

Figure 4 PLG protein expression in control liver, HCC-free and HCC-associated cirrhosis. The PLG protein levels were determined by immunohistochemistry (magnification x 20). A: Every protein level (IP score) per cirrhosis type is expressed on the ordinate. The mean value of protein level is shown as an horizontal bar. The samples abbreviations are the same as for Figure 3. Significant differences between A and PA ($^aP < 0.05$), between V and PV ($^bP < 0.05$), between A and V ($^cP < 0.05$). B-F: PLG immunostaining of hepatic sections corresponding to alcoholic HCC-free cirrhosis (B), alcoholic HCC-associated cirrhosis (C), control liver (D), HCV-related HCC-free cirrhosis (E) and HCV-related HCC-associated cirrhosis (F).

Table 4 Over-representation of functional subsets in our list of 70 transcripts

Cell proliferation[1] (O = 7; E = 2.66; $P = 0.01$[2])	Regulation of cell migration (O = 2; E = 0.15; $P = 0.009$)	Blood vessel development (O = 3; E = 0.41; $P = 0.007$)	Lipid metabolism (O = 9; E = 3.55; $P = 0.007$)	Antigen processing and presentation (O = 3; E = 0.25; $P = 0.001$)	Acute inflammatory response (O = 4; E = 0.58; $P = 0.002$)	NADH dehydrogenase activity (O = 3; E = 0.36; $P = 0.005$)	Oxidoreductase activity (O = 3; E = 0.25; $P = 0.001$)
JAG1 (Hs.590881[3])	JAG1 (Hs.590881)	JAG1 (Hs.590881)	CLU (Hs.436657)	HLA-A (Hs.181224)	CLU (Hs.436657)	NDUFA6 (Hs.274416)	CYP2E1 (Hs.12907)
IGFBP7 (Hs.479808)	PLG (Hs.143436)	PLG (Hs.143436)	CYP2J2 (Hs.152096)	HLA-B (Hs.77961)	ORM1 (Hs. 567311)	NDUFB10 (Hs.513266)	CYP2J2 (Hs.152096)
IL12RB2 (Hs.479347)		CUL7 (Hs.520136)	CYP3A4 (Hs.567254)	CD74 (Hs.591258)	ORM2 (Hs.522356)	NDUFS4 (Hs.528222)	CYP3A4 (Hs.567254)
PLG (Hs.143436)			HMGCS2 (Hs.59889)		STAT3 (Hs.463059)		
TIMP1 (Hs.522632)			APOB (Hs.120759)				
ARHGEF1 (Hs.438429)			HSD17B6 (Hs.524513)				
CD74 (Hs.591258)			DPM1 (Hs.301898)				
			LARGE (Hs.474667)				
			CD74 (Hs.591258)				

[1]This transcript subset coding for proteins with a related function was identified by gene ontology with the GOTM tool; [2]Significance of enrichment for the GO category between transcript number observed (O) and expected (E) in this cate gory; [3]Hs. number: Unique transcript identifier.

($P = 0.001$); (6) acute inflammatory response ($P = 0.002$); (7) NADH dehydrogenase activity ($P = 0.005$) and (8) oxidoreductase activity ($P = 0.001$).

Within our set of 70 transcripts, 23 transcripts with available information from Bibliosphere exhibited an etiology-associated level variation in HCC-free cirrhosis (Figure 5), but not in peritumoral cirrhosis (data not shown). This resulted from a variable extent of down-regulation (17/23 transcripts, 74%) in a single etiology or both. In turn, this down-regulation resulted, at least partly, from variable, etiology-dependent regulation of STAT-3 and its target genes (lower right area of Figure 5).

DISCUSSION

Our search first focused on the significance of

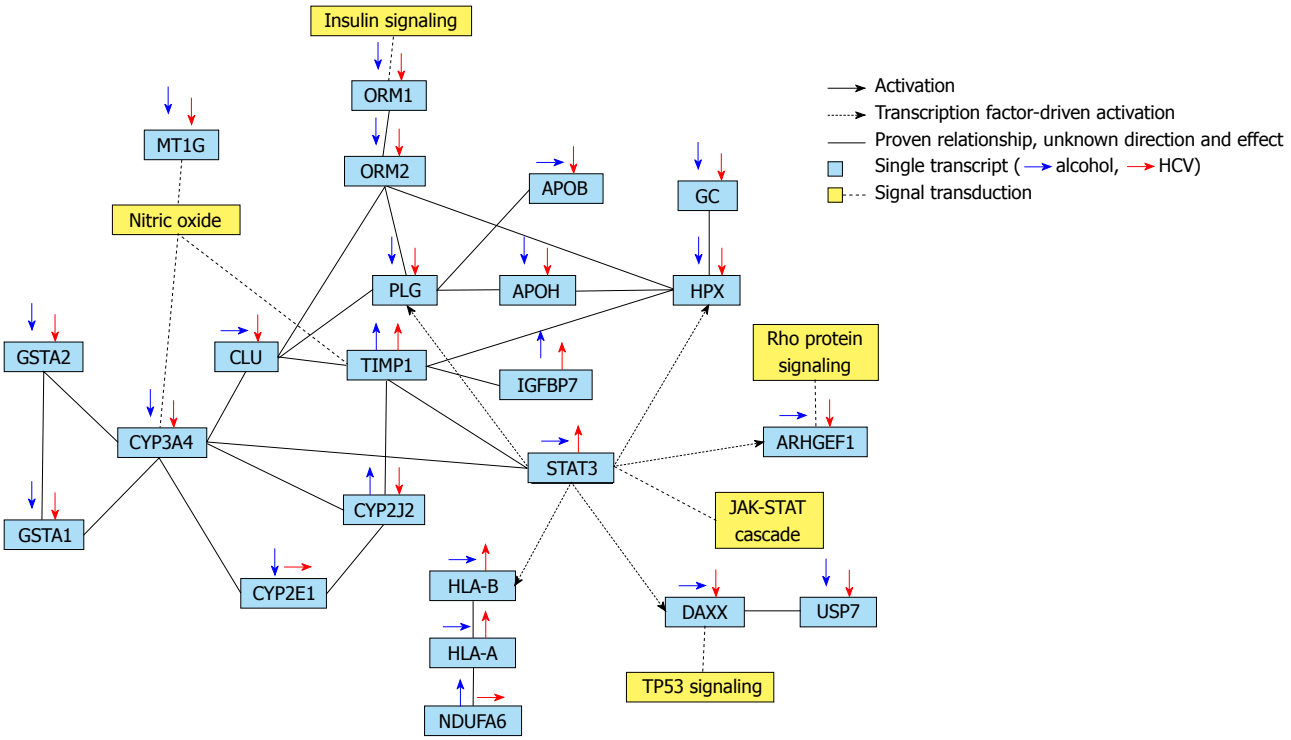

Figure 5 Networks of etiology-independent or dependent transcript regulations in HCC-free cirrhosis. From 70 transcripts with a significantly different expression between alcoholic-associated *vs* HCV-associated cirrhosis *vs* CLs ($P < 0.05$, as in Figure 3), 23 transcripts (boxes) associated with different pathways were identified in Bibliosphere. In a context of HCC-free cirrhosis, etiology-specific arrows (alcoholism, blue; HCV, red) above the transcript indicates the direction of regulation: upward, downward or horizontal arrow: up-regulation, down-regulation or unchanged regulation. For a transcript with an up- (down-) regulation in both etiologies, the highest (lowest) arrow indicates the most marked dysregulation.

dysregulations found in HCC-free cirrhosis. We investigated the influence of an HCC-free *vs* peritumoral environment of cirrhosis and we searched for early markers of HCC onset. The ideal study design would be to compare cirrhosis that develops into HCC and cirrhosis that does not develop into HCC in the follow-up of the disease, but such samples were very scarce because the follow-up among cirrhotic patients would be extremely long and difficult. So, we compared HCC-free and HCC-associated cirrhosis and we used a stringent selection of informative transcripts because sharp, timewise variations of potential markers of HCC occurrence were to be identified. We have shown that transcription dysregulation does exist in HCC-free cirrhosis. This is observed before any histologically detectable HCC nodule is seen, and hence supports the "field cancerization" model. Specifically, from our GOTM results, it seems plausible that malignant transformation of cirrhosis could be favored by abnormal expression of factors regulating cell migration, cell proliferation and blood vessel development. These dysregulations in cirrhosis mainly correspond to down-regulations, and they usually re-normalize or are even up-regulated in peritumoral cirrhosis. It remains to be determined how such transient down-regulations, which have been as yet unreported, contribute to HCC initiation.

We next carried out a similar search, further integrating alcoholism and HCV etiologies. A few, etiology-dependent markers for a high *vs* low risk of HCC development have been previously identified by others, but they relied on an unproven assignment of alcoholism or HCV patients to either risk group and they could not predict HCC occurrence in a timewise fashion[12]. We had previously reported that abnormalities of some transcript levels are observed to a different extent in HCC developed on alcoholic-associated cirrhosis *vs* HCV-associated cirrhosis, whereas they remain similar in peritumoral cirrhosis, thus indicating that these abnormalities are etiology-related in HCC tumors only[24]. In the same way, in the present study, we found transcript dysregulation only in HCC-free cirrhosis and not in peritumoral cirrhosis. We now document that histochemical evaluation of the PLG protein level confirms our results obtained at the transcriptional level. In contrast, for DPF2, dysregulation observed at both transcript and protein levels were in opposite directions, but discrepancies between transcript levels and protein levels have been previously noticed[26,27]. As markers of HCC occurrence are still very scarce[4,28], our observation on transcripts and proteins is of strong interest in early HCC diagnosis. This will need to be further evaluated in HCC-free/cirrhosis-free, fibrotic livers, with selection of marker combinations.

Some up- or down-regulations of transcription factors in HCC have already been documented[29], but the facts that dysregulation of STAT-3 and a related gene network take place in HCC-free cirrhosis, and in an etiology-dependent fashion, are novel findings. The JAK-STAT pathway is critical in the proinflammatory

cytokine-driven inflammatory response provided by hepatocytes[30] and it is tempting to speculate that a weakening of this defense in early cirrhosis may participate in HCC development. However, this mechanism appears unlikely. Indeed, our data were obtained by comparison of alcoholic *vs* HCV cirrhosis samples whose extent of inflammation was similar and still had different STAT-3 regulation. The HCV has a clear effect on the activity of STAT-3, but the meaning of this is controversial. Some studies show inhibition of STAT-3 activity[31] while others show activation of STAT-3[32-34]. Our data are in keeping with documented HCV proteins/STAT-3 interferences and STAT-3 activation in HCV-induced liver disease. STAT-3 directly affects cell proliferation, cell differentiation[35] and angiogenesis[32]. Moreover, STAT-3 and its targets are regulated in some cancers, such as breast and prostate cancer[34]. Thus, the dysregulation of the STAT-3 pathway which follows HCV infection may participate in HCC development at an early stage of hepatocyte dysplasia. In addition, recent reports have highlighted the potential of STAT-3 as a therapeutic target in different neoplasms[36,37].

In conclusion, our data point to major transcription dysregulations in HCC-free cirrhosis. These dysregulations often result from a transient dysregulation, and half are etiology-dependent. Our observations open new avenues for the follow-up of HCC-free cirrhosis because dysregulated transcripts or proteins may be appear like markers for the cirrhosis to HCC transition. In order to complement these results, studies performed at an earlier state before cirrhosis, i.e. on fibrosis samples are now under investigation.

ACKNOWLEDGMENTS

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COMMENTS

Background

Chronic viral hepatitis C (HCV) infection and alcoholism are two important causes of cirrhosis and hepatocellular carcinoma (HCC). Liver transcriptome analysis has resulted in the identification of genes with an aberrant expression according to different pathophysiological states. In the present work, we investigated whether some transcription dysregulations could be found in HCC-free cirrhosis in an etiology-dependent fashion. Furthermore, we searched and found transcription dysregulations that differentiate HCC-free cirrhosis from peritumoral cirrhosis.

Research frontiers

Numerous, genome-wide analyses of abnormal gene expression in HCC as compared to normal, control liver, have resulted in identification of gene sets with altered expression. Few similar studies have been done in HCC-free cirrhosis. In fact, viral etiologies have often been considered whereas abnormal gene expression in alcoholism-dependent HCC has received very little attention. Therefore, the impact of etiology still remains an important issue. We recently reported that a number of genome-wide abnormalities in alcoholism-associated *vs* HCV-associated HCC are etiology-dependent and some of them are of pathological relevance. Remarkably, the abnormal transcript levels that differentiate HCC nodules in an alcoholism-dependent *vs* HCV-dependent HCC can no longer discriminate between the two etiologies when transcripts are measured in the surrounding cirrhosis. Yet, any etiology-dependent abnormalities that could be observed in HCC-free cirrhosis would be of interest.

Applications

These data point to major transient transcription dysregulations in HCC-free cirrhosis. These observations open new avenues for the follow-up of HCC-free cirrhosis because dysregulated transcripts or proteins may be appear like markers for the cirrhosis to HCC transition.

Peer review

The aims of the study were to identify genes that were differentially expressed between HCC-free and HCC-related cirrhosis. The differentially expressed genes were further investigated to see if they were associated with alcoholism and HCV etiologies. The authors suggested that genes that were deregulated in HCC-free cirrhosis might serve as markers for the cirrhosis to HCC transition.

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ORIGINAL ARTICLES

Effects of ciglitazone and troglitazone on the proliferation of human stomach cancer cells

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Abstract

AIM: To determine the cytological and molecular effects of peroxisome proliferation-activated receptor (PPAR)-γ and PPAR-γ agonists on stomach cancer cells.

METHODS: To determine the proliferation-suppressive effects of troglitazone and ciglitazone, SNU-216 and SNU-668 stomach cancer cells were plated in media containing 40 μmol/L troglitazone and ciglitazone at a density of 1×10^4 cells/well. After 3, 5 and 7 d, the cells were counted with a hemocytometer. To assess the appearance of PPAR-γ, a reverse-transcription polymerase chain reaction analysis was performed. On day 7, Western blotting was used to determine the effects of troglitazone and ciglitazone on the expression of *p21* and phosphorylated-ERK (*pERK*) genes. Flow cytometry analysis was used to determine which portion of the cell cycle was delayed when troglitazone was used to suppress cell proliferation. In order to clarify the mechanism underlying the activity of troglitazone, microarray analysis was conducted.

RESULTS: PPAR-γ was manifested in both SNU-216 and SNU-668 cells. Ciglitazone and troglitazone suppressed cell growth, and troglitazone was a stronger suppressor of stomach cancer cells than ciglitazone, an inducer of cell cycle arrest in the G1 phase. SNU-668 cells were also determined to be more sensitive to ciglitazone and troglitazone than SNU-216 cells. When troglitazone and ciglitazone were administered to stomach cancer cells, levels of p21 expression were increased, but ERK phosphorylation levels were reduced. When GW9662, an antagonist of PPAR-γ, was applied in conjunction with ciglitazone and troglitazone, the cell growth suppression effect was unaffected. The gene transcription program revealed a variety of alterations as the consequence of troglitazone treatment, and multiple troglitazone-associated pathways were detected. The genes whose expression was increased by troglitazone treatment were associated with cell development, differentiation, signal transmission between cells, and cell adhesion, and were also associated with reductions in cell proliferation, the cell cycle, nuclear metabolism, and phosphorylation.

CONCLUSION: Troglitazone and ciglitazone suppress the proliferation of stomach cancer cells *via* a PPAR-γ-independent pathway.

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Key words: Peroxisome proliferating-activated receptor-γ; Ciglitazone; Troglitazone; Stomach cancer cells

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INTRODUCTION

The peroxisome proliferator-activated receptors (PPARs) are members of the family of nuclear receptors[1], which themselves constitute a group in the steroid/thyroid hormone/retinoid receptor superfamily. Their action mechanisms induce the formation of a heterodimer after PPAR unites with its ligand and retinoid X receptor (RXR) in the nucleus, which subsequently activates the manifestation of genetic DNA by working on the transcription factors of promoter sites. Thus far, three

subtypes, α, β/δ, and γ, have been identified, and an increasing quantity of research into PPAR-γ has been conducted since the initial detection of the composed ligand[2]. According to the research conducted over the last 10 years, PPAR-γ has been associated with novel functions in cell division and differentiation, functions crucial to inflammation, tissue resuscitation, vascular biology, cancer formation, and apoptosis[3]. As a result of these functions, the PPARs have been implicated as a treatment factor for diabetes mellitus, metabolic syndrome, atherosclerosis, and certain types of cancer[2,4]. PPAR-γ has been detected in a broad variety of cancers, including colon, breast, lung and prostate cancer. Ligands of PPAR-γ have been demonstrated to suppress the propagation of these cancers *in vitro*[5-8]. The results of this study suggest that many human malignant cancers may eventually be cured using PPAR-γ ligands. One well-known category of ligands is the thiazolidinediones (TZDs), which includes rosiglitazone, troglitazone, ciglitazone and 15-deoxy-prostaglandin-J2 (15d-PGJ2)[9].

Stomach cancer is one of the most common cancers worldwide. The condition is not so common in America and Europe, but is relatively common in Asia, and specifically in South Korea. A great many drugs already exist for treatment of stomach cancer, and these have already brought great improvements in survival rates and quality of life. However, there is currently no standard protocol by which personal sensitivity or resistance to treatment can be predicted. Lu *et al*[10] has previously reported that troglitazone suppresses stomach cancer *via* the activation of PPAR-γ, and in another study, it has been reported that stomach cancer is suppressed by PPAR-γ-ligand-mediated apoptosis[11].

The PPAR-γ ligand has two different pathways, one of which is PPAR-γ-dependent, and one PPAR-γ-independent[10,12-18]. The relationship between the independent pathway and stomach cancer has been confirmed, for example, by the finding that the 15d-PGJ2-induced suppression of colon cancer cells can be achieved *via* the manifestation of Kruppel-like factor 4 (KLF4)[16].

The principal objective of the present study was to determine the mechanism underlying the activity of PPAR-γ. After we confirmed the activation of PPAR-γ in two types of stomach cancer cells and administration of ciglitazone and troglitazone, both of which induce PPAR-γ activation, we were able to make an observation about cell proliferation, confirm the effects of PPAR-γ suppressors, and clarify any genetic alterations *via* the use of cDNA microarrays.

MATERIALS AND METHODS

Materials

We utilized troglitazone, ciglitazone, GW9662, propidium iodide, and dimethyl sulfoxide (DMSO) obtained from Sigma Co. (St. Louis, MO, USA), RPMI 1640, fetal bovine serum (FBS), 0.05% trypsin/0.02% EDTA, penicillin/streptomycin from Invitrogen Co. (Grand Island, NY, USA) and total-ERK, phosphorylated-ERK, and p21 antibody from Cell Signaling Technology Co. (Beverly, MA, USA). Troglitazone and ciglitazone solution was added at a concentration of 40 μmol/L per well. When adding the materials, we utilized DMSO solution and ensured identical conditions and DMSO concentration between the control and experimental groups.

Cultivation of cell strains

The SNU-216 and SNU-668 stomach cancer cell strains were obtained from the Korean Cell Bank (Seoul National University Hospital, Cancer Institute, Seoul, Korea) and were used as cultured. Cell culture was carried out at 37℃ in an atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Measurement of vegetative function

In order to determine the proliferation-suppressive effects of troglitazone and ciglitazone, after washing a growth phase cell strain, we separated cells with 0.05% trypsin/0.02% EDTA. These cells were mixed thoroughly and cultured for 24 h in six-well plates at a concentration of 1×10^4 cells/well. We verified the attachment of the cells to the plates, and then added 40 μmol/L troglitazone and ciglitazone to each 10% FBS medium. After 3, 5 and 7 d, we separated the proliferated cells with 0.05% trypsin/0.02% EDTA. These cells were counted with a hemocytometer and compared with the control group to assess the suppressive effects on cell growth.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

After washing the cultured cells with Hank's Buffered (or Balanced) Salt Solution (HBSS), we briefly mixed them with TRI Reagent (Sigma) and maintained them for 15 min at 4℃. We then mixed them one additional time with 200 μL chloroform and maintained them for an additional 5 min at 4℃. We then subjected the samples to centrifugation at a rate of 12000 rpm at 4℃, transferred the upper layer to a new tube and added an equal volume of isopropanol. This tube was maintained for 5 min at 4℃, and then centrifuged. The samples were dried and dissolved in diethylpyrocarbonate (DEPC)/distilled water, after washing the centrifuged pellets with DEPC/70% ethanol. The RNA was quantitated after determining the optical density at 260 nm, after which the reverse transcription reaction was conducted. Distilled water was added and settled with buffer (Promega, MO, USA) with 20 μmol/L dNTP, 0.25 μg oligo (dT) 15 primer, 5 U Avian Myoblastosis Virus (Promega), reverse transcriptase (Promega) and 2 μg RNA and DEPC. We established the total quantity at 20 μL and the reaction was performed for 60 min at 42℃.

We used an AccuPower PCR Premix (BIONEER, Seoul, Korea) kit. After the reverse transcription reaction was finished, we added 1 μL RT product and 10 pmol

sense and antisense primers into the tube provided in the kit. We established the total volume at 20 μL with distilled water and initiated the PCR.

Western blot analysis

After washing the cultured cells in HBSS, the cells were lysed in lysis buffer and then placed on ice for 30 min. The protein was extracted by centrifuging this solution. We then added 4 × loading buffer to the protein standard marker and to each protein (5 μg/μL), and then denatured them for 7 min at 95℃. Electrophoresis was conducted for 2 h at 100 V on 4% and 10% SDS-polyacrylamide gels. After electrophoresis, we placed the gel in transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, 20% methanol) for 15 min, and then transferred it to nitrocellulose (NC) membrane for 1 h at 20 V. In order to prevent non-specific antibody binding, we placed the NC membrane in blocking solution, 5% non-fat milk dissolved in Tris-buffered saline (TBS), and incubated the reactions with slight shaking. We then placed them into prepared primary antibody diluted 1/1000 with TBS/Tween buffer (TBS buffer with 0.02% Tween 20) including 5% non-fat milk, and then added it to the NC membranes overnight at 4℃. The samples were washed three times for 10 min each. We diluted the secondary antibody, anti-rabbit IgG in 5% non-fat milk to dilute it 1/1000, immersed the NC membrane in this solution with shaking for 90 min, and then washed the samples three times with TBS/Tween buffer for 10 min each. We subjected the NC membranes to enhance chemiluminescence (Amersham, Buckinghamshire, UK) for 5 min, exposed them to light for 10 min in a darkroom, and then detected the signals on the films. After visual certification of the luminosity and intensity of the bands, we quantified the intensity of the bands using a Gel Image Analysis System (UVItec, Cambridge, UK).

Cell cycle analysis *via* flow cytometry

We conducted flow cytometry analysis to determine which portion of the cell cycle was delayed when we used troglitazone to suppress the proliferation of SNU-216 and SNU-668 cells. Cultured SNU-216 and SNU-668 cell strains were washed in HBSS. We then added 40 μmol/L troglitazone, and fixed the cultures for 30 min with 70% ethanol at 4℃. After fixation, we degraded the RNA with RNase A (Sigma) and dyed the DNA with propidium iodide in order to prepare intercalated DNA. The cell cycles were compared and analyzed using a Becton-Dickinson FACStar Flow Cytometer and Becton-Dickinson Cell Fit Software (Becton-Dickinson, Erenbode, Belgium).

Illumina microarray

Microarray analysis was conducted using an Illumina BeadStation 500 X manual system, obtained from Microgen Co. (Seoul, Korea). We prepared the biotinylated cRNA with an Illumina Amplification Kit (Ambion, CA, USA), and refined it with an RNeasy Kit (Qiagen, CA, USA). Hybridization was conducted with a Sentrix HumanRef-8 Expression BeadChip system (Illumina, CA USA), which included approximately 24 000 probes, and conducted lavation in accordance with the manufactures instructions, followed by scanning with a confocal laser scanner (Nikon Precision Korea, Yong-In, Korea). We then conducted statistical analysis using Avadis Prophetic software, version 3.3 (Strand Genomics, Bangalore, India).

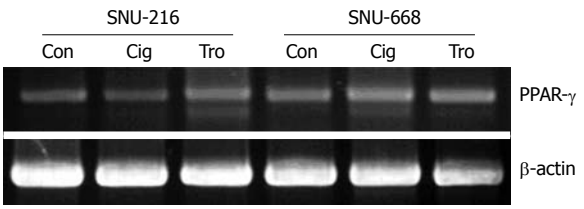

Figure 1 PPAR-γ expression was confirmed by RT-PCR in human gastric cancer cell lines (SNU-216 and SNU-668) treated with troglitazone (Tro) or ciglitazone (Cig) and the β-actin control (Con) is shown in the bottom panel.

Statistical analysis

One-way analysis of variance and Fisher's LSD test were used to compare statistical differences between each group, and a P value < 0.05 was considered significant for all statistical analyses.

RESULTS

The manifestation of PPAR in SNU-216 and SNU-668 stomach cancer cells

As a result of the manifestation of PPAR-γ in stomach cancer cells SNU-216 and SNU-668 using RT-PCR, both cells were confirmed to be positive, and no significant differences were detected (Figure 1). Additionally, the difference in the degree of manifestations was not significantly different when troglitazone and ciglitazone were applied at 40 μmol/L for 7 d, and the manifestations of PPAR-γ were assessed *via* RT-PCR (Figure 1).

Changes in cell morphology by settlement of troglitazone and ciglitazone

We observed cell morphology after exposing SNU-216 and SNU-668 cells to 40 μmol/L troglitazone and ciglitazone for 7 d. The SNU-216 cells showed no significant morphological changes after treatment with the two compounds. On the contrary, the SNU-668 cells demonstrated morphological changes after 2 d troglitazone, but not ciglitazone, treatment; the cells were lengthened at both ends, and assumed a spindle-type morphology (Figure 2).

Troglitazone- and ciglitazone-induced inhibition of SNU-216 and SNU-668 cell proliferation

In order to assess the suppressive effects of troglitazone and ciglitazone on the proliferation of SNU-216 and

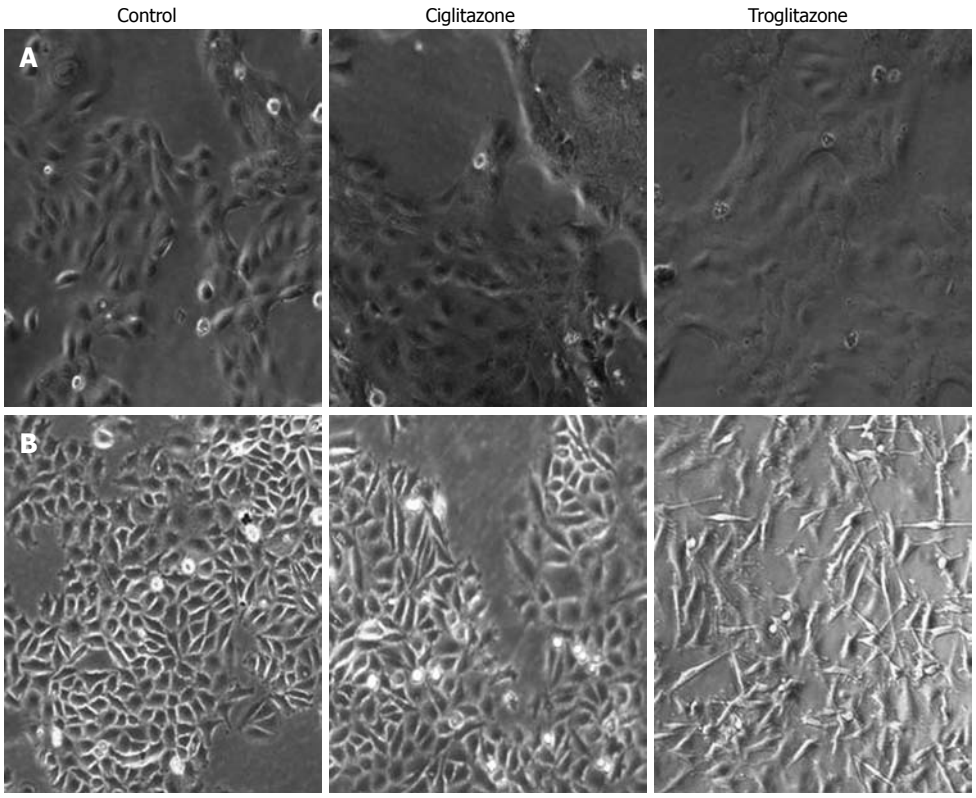

Figure 2 Change in cell morphology after treatment with troglitazone or ciglitazone. There were more significant differences in SNU-668 (B) than SNU-216 (A) cells, as shown by inverted microscopy (original magnification, x 100).

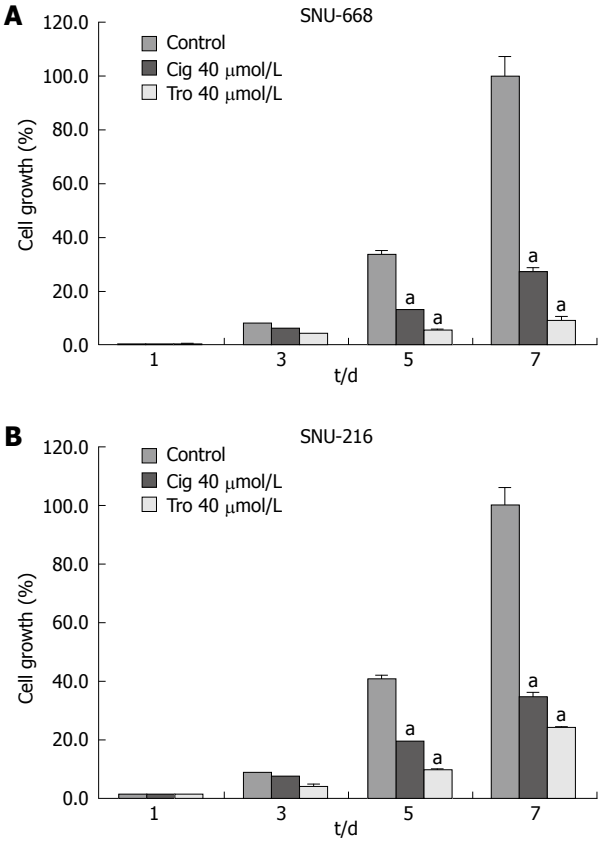

Figure 3 Growth inhibition by troglitazone or ciglitazone in human gastric cancer cell lines. There was a more significant increase of suppressive effect in SNU-668 (A) than SNU-216 (B) cells compared with the control group. $^{a}P < 0.05$ *vs* control group.

SNU-668 cells, we cultured the cells for 24 h on six-well plates at 1×10^4 cells/well. We added troglitazone and ciglitazone to the medium, and cultured the cells again. We counted the number of cells at 3, 5 and 7 d after culturing. The growth rate of the SNU-216 cells was reduced at 3, 5 and 7 d after troglitazone treatment, and the cell count percentages were 45%, 24% and 24%, as compared with the control group. The growth rate of the SNU-668 cells was reduced on the same days by troglitazone treatment, by 49%, 15% and 9%. However, with ciglitazone treatment, the growth rate of the SNU-688 strain was reduced less profoundly, and the percentages of the cell count were 77%, 38% and 27%. As a result, troglitazone appeared to exert a more profound suppressive effect than did ciglitazone (Figure 3). Ciglitazone and troglitazone treatment did not significantly affect SNU-216 and SNU-668 cell death (Figure 4).

Cell cycle analysis using flow cytometry

We assessed the cell cycles of the two groups. One group was SNU-216 and SNU-668 cells cultured in media to which 40 μmol/L troglitazone and ciglitazone was added, and the other group was a control group that was cultured without any drug treatment. In the SNU-216 cells, each of the G0/G1 phases were 77%, 78% and 79%, the S phases were all 11%, and the G2/M phases were 12%, 11% and 10%. However, in the SNU-668 cells, the G0/G1 phases of each group were 73%, 76% and 86%, the S phases were 11%, 11% and 8%, and the G2 phases were 16%, 12% and 6% (Figure 5).

Effect of troglitazone and ciglitazone on expression of p21 and pERK genes in SNU-216 and SNU-668 cells

We cultured SNU-216 and SNU-668 cells at a

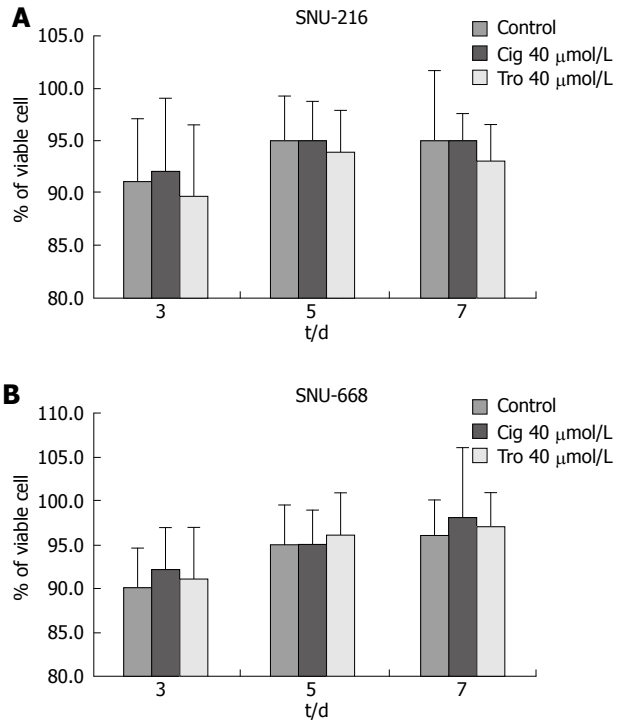

Figure 4 Cell viability measured by hemocytometry. Viability of the troglitazone- or ciglitazone-treated cells was decreased more than that of the control group, with no significant difference between the two cell lines.

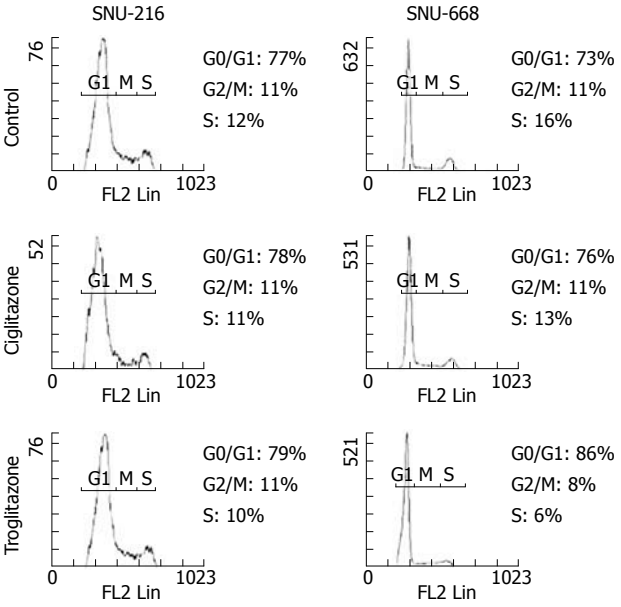

Figure 5 Effects of troglitazone and ciglitazone on cell cycle distribution measured by flow cytometry. It shows meaningful arrest during G2/M and S phase in SNU-668 treated with triglitazone.

concentration of 1×10^4 cells/well on six-well plates for 24 h, and added troglitazone and ciglitazone at levels of up to 40 μmol/L for 7 d. On day 7, we conducted Western blotting after extracting the proteins with from the cultured cells for 7 d, and assessed the density of the bands *via* image analysis. As a result of the expression of p21, we noted no significant interval changes before and after drug treatment in SNU-216 cells. In SNU-668 cells, we noted an increase of approximately 2.8-fold

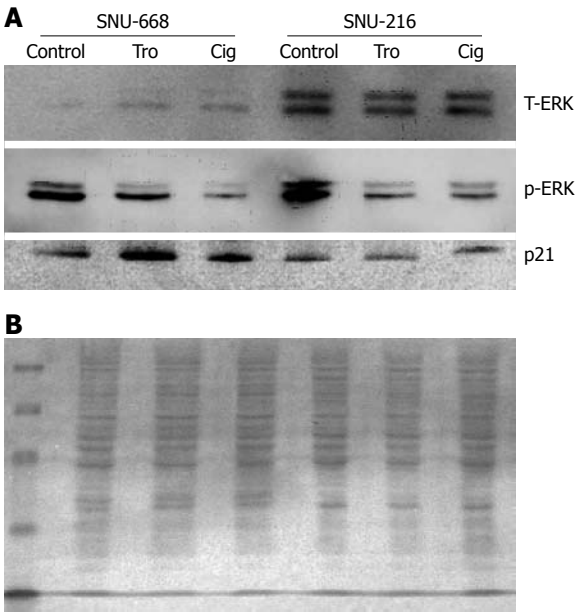

Figure 6 Western blot analysis for the expression of total-ERK, p-ERK and p21. A: There was a significant decrease in phosphorylation of ERK and increased expression of p21 in SNU-668 cells with ciglitazone or troglitazone treatment. B: Ponceau S protein staining of the membrane of SNU-216 and SNU-668 cells to ensure equal loading of protein in the sample.

with troglitazone, and a 1.6-fold increase with ciglitazone. Phosphorylation of ERK was significantly reduced by ciglitazone and troglitazone (Figure 6).

Influence of PPAR-γ antagonists on the suppressive effects of troglitazone and ciglitazone on cell growth

In order to evaluate the association between the suppressive effects of cell growth with identical levels of troglitazone, ciglitazone and PPAR-γ activation, we confirmed the effects of GW9662[19], which has been previously identified as a selective PPAR-γ suppressor. After culturing the solution at 1×10^4 cells/well in six-well plates, we added antagonist 5 μmol/L GW9662, 5 μmol/L GW9662 + 40 μmol/L ciglitazone, 5 μmol/L GW9662 + 40 μmol/L troglitazone to each medium, and counted the cells 3, 5, and 7 d later. The suppressive effects of 40 μmol/L troglitazone and ciglitazone was not influenced by treatment with 5 μmol/L GW9662 (Figure 7).

Changes in gene expression

To elucidate the mechanism underlying the activity of troglitazone, we evaluated the troglitazone-induced changes in gene expression, *via* the microarray technique. We verified these changes, and found that expression of 388 genes was increased by more than two-fold, and 466 were reduced by more than two-fold. According to the analysis of genetic functions with genetic manifestation by PANTHER (Protein Analysis Through Evolutionary Relationsips) Classification System (SRI international, CA, USA), cell cycle, DNA metabolism, somatic cell division, replication, and DNA repair were suppressed to a significant degree. However, signal transduction and homeostasis were increased (Table 1). In an effort to determine the categories of biological processes affected

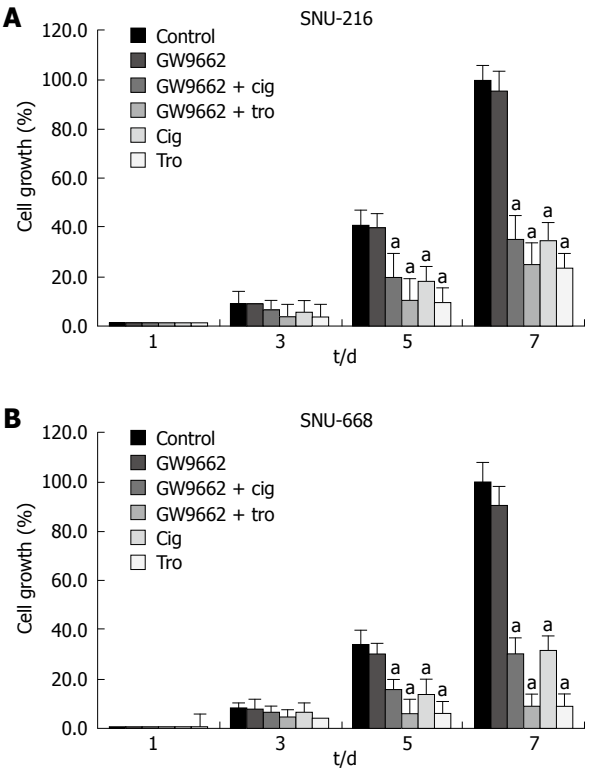

Figure 7 Effect of GW9662 on the inhibition of cell proliferation. GW9662, a selective PPAR-γ suppressor, had no effect on ciglitazone- or troglitazone-induced inhibition of cell proliferation. $^{a}P < 0.05$ *vs* control group.

by troglitazone treatment, we conducted functional annotation analyses using DAVID bioinformatic tools[20], with the genes whose expression was altered more than two-fold. The results of this analysis reveal a distinct distribution of biological processes between these genes (Tables 2 and 3). The expression of genes associated with signal transmission for cell communication, growth, differentiation, and cell adhesion was increased. Additionally, expression of genes associated with cell proliferation, the cell cycle, nuclear metabolism and phosphorylation was also reduced. The functional differences between the increased and reduced genes were confirmed by the changes in the KEGG pathway, as detected by DAVID analysis. The pathway underlying the reductions in the levels of these genes was associated with the cell cycle, DNA polymerase, and purine and pyrimidine metabolism, the effects of which on the genes with increased expression was not confirmed (Table 4).

DISCUSSION

PPAR-γ is manifested in a variety of tissues and cancer cells[21], and the ligands that activate PPAR-γ are currently being studied as a possible novel therapeutic modality[19,22-26]. The PPAR-γ ligand generally reduces the survival rate of cancer cells *via* differentiation, apoptotic induction, and changes in genes or proteins associated with entrance into the G1/S phase[15,27]. Some reports have suggested that stomach cancer cells manifest PPAR-γ and are suppressed by PPAR-γ ligands[11]. Recently, another study has demonstrated that troglitazone, a PPAR-γ ligand, may prove useful in preventive medicine, and this effect appears to occur in a PPAR-γ-dependent manner[10].

Table 1 Alteration of biological processes in SNU-668 cells treated with troglitazone as compared with controls

Biological process	Number	Over/under	*P* value
Cell cycle	116	-	3.510E-13
DNA metabolism	57	-	1.150E-09
Mitosis	38	-	1.060E-07
DNA replication	38	-	7.350E-06
DNA repair	23	-	1.110E-03
Cell surface receptor mediated signal transduction	52	+	1.850E-03
Cytokinesis	10	-	3.280E-03
Chromosome segregation	14	-	3.300E-03
Homeostasis	14	+	9.100E-03
Signal transduction	133	+	1.230E-02
Transport	41	+	1.740E-02
Nucleoside, nucleotide and nucleic acid metabolism	165	-	1.800E-02

The results of our study show that ciglitazone and troglitazone suppress the proliferation of stomach cancer cells *via* G1 phase arrest. The arrest of G1 phase has been reported in colon cancer cells as the result of PPAR-γ activation[12]. In the present study, each of the stomach cancer cell types that showed p53 mutations was suppressed more strongly by troglitazone than by ciglitazone, and this effect was detected more prominently in the SNU-668 cells than in the SNU-216 cells. The SNU-668 cells demonstrated p53 and ras mutations, and it will be necessary in future studies to clarify the relationship between these results and the medical mechanisms underlying them.

Few investigations have thus far addressed the induction mechanism by which stomach cancer cell proliferation is suppressed by troglitazone. As the results of some studies have suggested that PPAR agonists induce apoptosis in cancer cells, the observed cell-proliferation-suppressive effects may derive from a reduction in cell consistency. This indicates that the reduction in cells as the result of apoptosis is not very relevant to the aforementioned cell-proliferation-suppressive effects.

Jung *et al*[28] have evaluated the reaction of the PPAR-γ agonist pathway on PPAR-γ. For example, troglitazone suppresses the proliferation of colon cancer cells, induces apoptosis, and induces the growth response-1 gene in early phase. These processes are activated downstream of the suppression in a one-by-one fashion, *via* a PPAR-γ-independent pathway[29]. Chintharlapalli *et al*[17,18] have reported that an inductive chemical compound associated with CDDO induces both PPAR-γ-dependent caveolin manifestation and a PPAR-γ-independent induction of apoptosis. In our study, we determined that this mechanism occurs *via* an independent pathway, as suppression of the PPAR-γ agonist was not suppressed by PPAR-γ antagonists.

We utilized different concentrations of troglitazone to induce growth suppression, as has been done for

Table 2 Biological process of up-regulated genes by triglitazone in SNU-668 cells

Term	Count	*P* value	Genes
Negative regulation of cellular process	29	2.26E-04	ANGPTL4, ZFHX1B, MXD4, CXCL1, GPNMB, CDKN1A, IRF1, SESN2, JAZF1, FTH1, IL6, SVIL, JARID1B, MN1, DDIT3, PPP1R15A, IER3, ARHGEF2, TNFAIP3, RHOB, PRDM1, QSCN6, BNIP3L, MAP4, NRG1, EREG, FAIM3, IL8, FST
Response to chemical stimulus	20	1.91E-04	CCL20, PLD1, STC2, APOE, DEFB1, PLAU, CXCL1, SEMA3C, SRXN1, CCL5, SOD2, HSPA6, STC1, CXCL2, NDRG1, DNAJB2, IL8, MVP, ECGF1, ARNT2
Chemotaxis	8	0.009882707	CCL20, PLD1, CXCL2, CXCL1, PLAU, IL8, ECGF1, CCL5
Negative regulation of cell proliferation	9	0.009171778	FTH1, IL6, MXD4, EREG, CXCL1, IL8, GPNMB, CDKN1A, QSCN6
Programmed cell death	22	0.002544517	SQSTM1, TNFRSF21, PPP1R15A, APOE, ANGPTL4, IER3, TNFAIP3, RHOB, TNFRSF10A, ROCK1, HIPK2, CDKN1A, BNIP3L, C10orf97, IL6, RRAGC, APP, PHLDA2, RIPK2, FAIM3, IL24, TRIB3
Development	62	9.62E-06	SERPINE2, CXCL1, PHGDH, EPC1, MSX1, IL6, SLC3A2, COL7A1, IFRD1, NAV1, LIF, IER3, ARHGEF2, RHOB, DHRS9, PKD1, VAT1, SEMA3C, QSCN6, ANPEP, LTBP4, EDG3, SOX9, SQSTM1, PTGS2, IGF2BP3, FBN1, DNER, IGFBP5, CTGF, ANGPTL4, APOE, ZFHX1B, CSPG2, IL11, KLF6, RRAGC, S100P, NDRG1, SVIL, PNMA1, DACT1, CYP1B1, WNT5A, MAFB, PDLIM7, LAMA4, CRMP1, PAPPA, CAMTA1, RUNX1, KRTHA4, EREG, GPR56, NRG1, FST, IL8, DCN, ECGF1, ARNT2, NRP1, ITGA2
Organelle organization and biogenesis	28	0.005845300	WASPIP, APOE, HIST1H1C, CXCL1, EPC1, SVIL, MICAL1, H2BFS, HIST2H2AA, FMNL2, TINF2, PLEC1, TUBA1, HIST2H2BE, ARHGEF2, RHOF, ROCK1, TMOD1, HIST1H2BK, RHOB, SLC22A4, FSCN1, HIST1H2BD, ATG4A, KIF1A, MAP4, ECGF1, KLHL5
Inflammatory response	12	0.002509065	CCL20, PTGS2, CXCL2, PLA2G4C, CEBPB, EDG3, CXCL1, RIPK2, IL8, TNFAIP6, IRAK2, CCL5
Organ development	21	0.003134980	FBN1, ANGPTL4, PDLIM7, RHOB, PHGDH, IL11, ANPEP, MSX1, IL6, KLF6, SVIL, EREG, GPR56, SOX9, DCN, IL8, ECGF1, CYP1B1, NRP1, ITGA2, IFRD1
Transcription from RNA polymerase Ⅱ promoter	17	0.024568720	SQSTM1, TRAK1, TSC22D1, DDIT3, MAFF, MXD4, IRF1, PRDM1, ATBF1, JAZF1, SOD2, FOXF1, NFIL3, RUNX1, STAT5A, CEBPB, SOX9
Cellular morphogenesis	12	0.016732354	KLF6, RRAGC, CTGF, IGFBP5, APOE, LTBP4, ARHGEF2, SLC3A2, VAT1, NRP1, QSCN6, EPC1
Blood vessel morphogenesis	7	0.002345097	ANPEP, ANGPTL4, EREG, RHOB, IL8, ECGF1, NRP1
Response to wounding	20	2.23E-04	CCL20, PTGS2, CTGF, PLA2G4C, PLAU, CXCL1, TNFAIP6, CCL5, SOD2, IL11, CXCL2, CEBPB, EREG, EDG3, RIPK2, FAIM3, IL8, SERPINE1, IRAK2, ITGA2
Apoptosis	22	0.002437855	SQSTM1, TNFRSF21, PPP1R15A, APOE, ANGPTL4, IER3, TNFAIP3, RHOB, TNFRSF10A, ROCK1, HIPK2, CDKN1A, BNIP3L, C10orf97, IL6, RRAGC, APP, PHLDA2, RIPK2, FAIM3, IL24, TRIB3
Cell-cell signaling	24	2.63E-04	CCL20, PBEF1, STC2, APOE, STX1A, LIF, GDF15, MAOA, TNFAIP6, GRB10, CCL5, IL11, STC1, GABARAPL1, IL6, LTBP4, EREG, GPR56, EFNA1, FST, IL8, ECGF1, NRP1, WNT5A
Response to stress	35	0.003636369	SQSTM1, CCL20, PTGS2, ERRFI1, PLA2G4C, CTGF, ANGPTL4, APOE, CXCL1, CD83, SRXN1, TNFAIP6, CCL5, IL11, SOD2, HSPA6, IL6, CD55, CEBPB, DNAJB2, SERPINE1, CFB, PPP1R15A, DDIT3, DEFB1, PLAU, CXCL2, EREG, EDG3, RIPK2, FAIM3, IL8, ARNT2, IRAK2, ITGA2
Vasculature development	7	0.002345097	ANPEP, ANGPTL4, EREG, RHOB, IL8, ECGF1, NRP1
Regulation of cell proliferation	16	6.45E-04	PBEF1, LIF, MXD4, ARHGEF2, CXCL1, GPNMB, CDKN1A, QSCN6, IL11, IRS2, FTH1, IL6, EREG, EDG3, IL8, NRP1
Cell organization and biogenesis	47	0.001133913	SQSTM1, TRAK1, WASPIP, STX3A, IGFBP5, CTGF, APOE, HIST1H1C, CXCL1, DNAJC12, EPC1, GABARAPL1, KLF6, RRAGC, SVIL, LARP6, MICAL1, HIST2H2AA, H2BFS, SLC3A2, FMNL2, TINF2, PLEC1, STX1A, TUBA1, DUSP16, HIST2H2BE, STX11, RHOF, ARHGEF2, RHOB, HIST1H2BK, TMOD1, ROCK1, SLC22A4, VAT1, FSCN1, QSCN6, BET1, HIST1H2BD, ATG4A, KIF1A, LTBP4, MAP4, ECGF1, KLHL5, NRP1
Cell differentiation	21	0.001730483	SQSTM1, PTGS2, APOE, ANGPTL4, PDLIM7, SERPINE2, RHOB, DHRS9, PAPPA, IL11, ANPEP, IL6, KLF6, NDRG1, LTBP4, EREG, NRG1, ECGF1, ARNT2, NRP1, IFRD1
Behavior	11	0.004559090	CCL20, PLD1, CXCL2, APOE, MAOA, PI3, CXCL1, PLAU, IL8, ECGF1, CCL5
Angiogenesis	7	0.001885665	ANPEP, ANGPTL4, EREG, RHOB, IL8, ECGF1, NRP1
Nucleosome assembly	7	0.007444314	HIST1H2BD, HIST2H2AA, HIST2H2BE, H2BFS, HIST1H1C, HIST1H2BK, SLC22A4
Cell adhesion	24	0.00542196	CLDN12, CTGF, LAMA4, CSPG2, RHOB, PKD1, CLDN1, TNFAIP6, CCL5, TPBG, KIAA0319, APP, ITGA5, LPP, GPR56, VTN, NID2, MYO10, IL8, COL7A1, MSLN, NRP1, ITGA3, ITGA2
Morphogenesis	27	8.06E-05	IGF2BP3, IGFBP5, CTGF, ANGPTL4, APOE, EPC1, MSX1, KLF6, RRAGC, SLC3A2, WNT5A, IER3, ARHGEF2, DHRS9, PKD1, RHOB, VAT1, QSCN6, ANPEP, LTBP4, EREG, EDG3, IL8, DCN, ECGF1, NRP1, ITGA2
Taxis	8	0.009882707	CCL20, PLD1, CXCL2, CXCL1, PLAU, IL8, ECGF1, CCL5
Glutamine family amino acid metabolism	5	0.007880038	GLS, GCLC, GFPT2, ASS, ASNS

studies of insulin resistance[30]. A similar technique has also been utilized to induce growth suppression in human colon cancer cells, as reported by Sarraf *et al* (1998)[13]. This suggests that the clinical concentrations of troglitazone should effectively suppress the growth of stomach cancer cells.

The intracellular mechanisms relevant to the growth suppression effects of ciglitazone and troglitazone remain to be elucidated; however, Western blotting results have verified that ERK phosphorylation is suppressed by troglitazone and ciglitazone, and the noted increase in the manifestation of p21 is more marked with troglitazone. We conclude that ciglitazone and troglitazone are associated with the suppression of cell growth. Our microarray analysis results showed that troglitazone induces not only the expression

Table 3 Biological process of down-regulated genes by troglitazone in SNU-668 cells

Term	Count	*P* value	Genes
Mitotic sister chromatid segregation	10	1.61E-10	CDCA5, KNTC2, CNAP1, DLG7, NUSAP1, HCAP-G, SMC4L1, CENPE, SMC2L1, ESPL1
Phosphorylation	32	6.36E-04	CDK6, CCL2, BUB1, INHA, CDC7, PKMYT1, GSG2, PBK, BUB1B, CHEK1, LOC91461, MASTL, WEE1, AURKB, MXRA5, PKN3, VRK1, CIT, PRKCE, PLK2, MELK, PASK, TTK, NEK2, EME1, NUAK2, CDC2, CDK2, CDK4, MAP2K6, PLK1, PLK4
Mitotic chromosome condensation	6	2.73E-06	CDCA5, CNAP1, NUSAP1, HCAP-G, SMC4L1, SMC2L1
Mitosis	44	4.68E-37	KIF2C, CENPF, BUB1, SPAG5, DLG7, ACTG1, PKMYT1, CDC6, HCAP-G, MAD2L1, CCNB1, PBK, CCNF, BUB1B, ESPL1, CCNB2, WEE1, CNAP1, KIF23, SGOL1, CIT, CDC25A, TPX2, ASPM, SMC4L1, SMC2L1, MPHOSPH1, TTK, NEK2, CDC2, CDK2, CCNA2, CDC25C, PTTG1, KNTC1, KIF15, PLK1, CDCA5, CDC20, KNTC2, NUSAP1, ANLN, UBE2C, CENPE
Phosphoinositide-mediated signaling	13	8.33E-08	CKS2, RFC4, HMGB2, HIST1H4C, TYMS, SPAG5, BUB1B, TOP2A, KNTC2, ZWINT, PCNA, UBE2C, FEN1
Response to DNA damage stimulus	43	1.78E-25	RFC4, CHAF1B, FANCL, POLD3, NUDT1, UHRF1, EXO1, CHEK1, LIG1, BRCA1, TOP2A, POLE2, RAD54L, GTSE1, POLE, PCNA, RAD51, MDC1, UIP1, RAD51AP1, HMGB2, RPA1, TYMS, POLQ, NEIL3, CCNA2, CHAF1A, PTTG1, MAP2K6, RFC5, FANCG, RPA3, FANCB, XRCC3, H2AFX, TOPBP1, RECQL4, BLM, RAD51C, POLD1, FANCD2, APEX2, FEN1
Traversing start control point of mitotic cell cycle	5	2.73E-06	CDC7, CDC6, CDC2, CDK2, CDC25C
Regulation of DNA replication	5	2.14E-04	GMNN, CDC6, CDK2, CDT1, PCNA
Regulation of DNA metabolism	7	6.56E-05	BRCA1, GMNN, CDC6, CDK2, CDT1, PCNA, RAD51
Phosphate metabolism	35	1.83E-03	CDK6, CCL2, BUB1, INHA, CDC7, PKMYT1, GSG2, CDKN3, PBK, BUB1B, CHEK1, LOC91461, MASTL, WEE1, AURKB, MXRA5, PKN3, VRK1, CIT, CDC25A, PRKCE, MELK, PLK2, PASK, TTK, NEK2, EME1, NUAK2, CDC2, CDK2, CDC25C, CDK4, MAP2K6, PLK4, PLK1
Organelle organization and biogenesis	44	7.49E-08	CKS2, KRT8, PRC1, KIF2C, CENPF, SUV39H1, CHAF1B, SPAG5, EZH2, KIF14, HCAP-G, KIF4A, DIAPH3, BUB1B, ESPL1, BRCA1, ACD, HIST1H2BH, CNAP1, KIF23, CBX1, ZWINT, GTSE1, KIF11, SMC4L1, SMC2L1, EXOSC2, MPHOSPH1, STMN1, TTK, HMGB2, HIST1H4C, CENPA, KIF20A, CHAF1A, KIF15, CDCA5, H2AFX, KNTC2, MGC39900, NUSAP1, UBE2C, CENPE, C9orf48
Organelle localization	6	1.62E-06	CENPF, CDCA5, DLG7, NUSAP1, CENPE, ESPL1
Microtubule-based process	22	7.74E-11	STMN1, CKS2, PRC1, KIF2C, TTK, SPAG5, KIF14, KIF4A, KIF20A, BUB1B, ESPL1, KIF15, KNTC2, KIF23, NUSAP1, ZWINT, GTSE1, KIF11, UBE2C, CENPE, C9orf48, MPHOSPH1
Sister chromatid segregation	10	2.68E-10	CDCA5, KNTC2, CNAP1, DLG7, NUSAP1, HCAP-G, SMC4L1, CENPE, SMC2L1, ESPL1
Regulation of cyclin dependent protein kinase activity	10	1.66E-07	CHEK1, CKS2, CDK5RAP3, PKMYT1, BCCIP, CDC6, CDC25A, CDKN3, CCNA2, CDC25C
Deoxyribonucleotide biosynthesis	3	6.19E-03	RRM2, TYMS, DTYMK
Response to stress	56	1.70E-09	CCL2, EXO1, LIG1, RAD54L, BST1, GTSE1, POLE, MDK, TYMS, POLQ, NEIL3, CCNA2, PTTG1, RFC5, FANCG, RPA3, FANCB, XRCC3, H2AFX, RECQL4, HSPA2, FANCD2, APEX2, RFC4, CHAF1B, INHA, FANCL, POLD3, NUDT1, UHRF1, FOS, CHEK1, BRCA1, TOP2A, GP1BB, POLE2, PCNA, RAD51, MDC1, UIP1, RAD51AP1, FOXM1, HMGB2, RPA1, CHAF1A, MAP2K6, CFH, TOPBP1, CD14, NR3C1, BLM, CLEC2D, RAD51C, POLD1, FEN1, PRDX2
Metaphase plate congression	3	2.55E-03	CENPF, CDCA5, CENPE
Regulation of transferase activity	12	1.44E-04	CHEK1, CKS2, CDK5RAP3, TPD52L1, PKMYT1, BCCIP, CDC6, CDC25A, CDKN3, CCNA2, CDC25C, RGS4
DNA strand elongation	3	4.18E-03	PRIM1, RFC4, RFC3
Cell proliferation	37	9.94E-10	CKS2, CDK6, KIF2C, CENPF, BUB1, CDC7, SKP2, DLG7, CDC6, CDKN3, DTYMK, BUB1B, CHEK1, STIL, BRCA1, CDKN2C, CDC25A, TPX2, PCNA, IFITM1, TTK, CDK5RAP3, MDK, MKI67, CDK2, CDC25C, ADAMTS1, TSPAN3, CDK4, CDCA7, CYR61, KIF15, CDCA7L, HDGF, PLK1, E2F1, UBE2C
Establishment of organelle localization	6	4.57E-07	CENPF, CDCA5, DLG7, NUSAP1, CENPE, ESPL1
Cell organization and biogenesis	59	6.71E-06	PRC1, KIF2C, SUV39H1, DLG7, HCAP-G, DIAPH3, ESPL1, ACD, CNAP1, KIF23, CBX1, THOC4, KIF11, GTSE1, EXOSC2, MPHOSPH1, STMN1, IL17RB, CENPA, CYR61, H2AFX, KNTC2, NUSAP1, HNRPA1, C9orf48, SLC25A10, NUP107, CKS2, KRT8, CENPF, CHAF1B, SPAG5, EZH2, KIF14, KIF4A, BUB1B, BRCA1, HIST1H2BH, TRIP6, ZWINT, SMC4L1, SMC2L1, TTK, KAZALD1, HMGB2, HIST1H4C, RANBP1, IGFBP3, TMEM97, KIF20A, CHAF1A, SORT1, KIF15, PPIH, CDCA5, MGC39900, UBE2C, WISP2, CENPE
Regulation of kinase activity	12	1.35E-04	CHEK1, CKS2, CDK5RAP3, TPD52L1, PKMYT1, BCCIP, CDC6, CDC25A, CDKN3, CCNA2, CDC25C, RGS4
DNA repair	40	3.75E-24	RFC4, CHAF1B, FANCL, POLD3, NUDT1, UHRF1, EXO1, CHEK1, LIG1, BRCA1, TOP2A, POLE2, RAD54L, POLE, PCNA, RAD51, MDC1, UIP1, RAD51AP1, HMGB2, RPA1, TYMS, POLQ, NEIL3, CHAF1A, PTTG1, RFC5, FANCG, RPA3, FANCB, XRCC3, H2AFX, TOPBP1, RECQL4, BLM, RAD51C, POLD1, FANCD2, APEX2, FEN1
DNA recombination	11	3.91E-06	CHEK1, LIG1, XRCC3, H2AFX, RPA1, BLM, RAD51C, RAD54L, RAD51, RAD51AP1, EXO1

Protein complex assembly	14	7.55E-03	CENPF, HMGB2, CHAF1B, HIST1H4C, SLC7A6, MPP2, CENPA, CHAF1A, KNTC1, PPIH, H2AFX, HIST1H2BH, RAD51, CENPE
Establishment of chromosome localization	4	8.79E-05	CENPF, CDCA5, DLG7, CENPE
Chromosome segregation	15	1.42E-14	CENPF, DLG7, HCAP-G, PTTG1, SGOL2, ESPL1, CDCA5, KNTC2, CNAP1, SGOL1, NUSAP1, CDCA1, SMC4L1, SMC2L1, CENPE
Chromosome organization and biogenesis	19	9.65E-05	CENPF, SUV39H1, HMGB2, CHAF1B, HIST1H4C, EZH2, HCAP-G, CENPA, CHAF1A, CDCA5, ACD, H2AFX, CNAP1, HIST1H2BH, CBX1, NUSAP1, SMC4L1, SMC2L1, CENPE
DNA integrity checkpoint	6	2.66E-05	CHEK1, CDC6, GTSE1, CDT1, CCNA2, CDC45L
Chromosome localization	4	8.79E-05	CENPF, CDCA5, DLG7, CENPE
Spindle organization and biogenesis	11	4.01E-12	CKS2, STMN1, PRC1, TTK, KNTC2, SPAG5, KIF23, ZWINT, KIF11, UBE2C, BUB1B
Protein amino acid phosphorylation	30	8.41E-05	CDK6, CCL2, BUB1, CDC7, PKMYT1, GSG2, PBK, BUB1B, LOC91461, MASTL, CHEK1, WEE1, AURKB, MXRA5, PKN3, VRK1, CIT, PRKCE, PLK2, MELK, PASK, TTK, NEK2, NUAK2, CDC2, CDK2, CDK4, MAP2K6, PLK1, PLK4
Chromosome condensation	6	9.81E-06	CDCA5, CNAP1, NUSAP1, HCAP-G, SMC4L1, SMC2L1
Cell cycle	97	2.18E-51	PRC1, KIF2C, CDC7, GSG2, CDKN3, HCAP-G, ESPL1, LIG1, MCM5, TPD52L1, CNAP1, KIF23, RAD54L, GTSE1, STMN1, NEK2, E2F2, CDK4, SGOL2, KNTC1, PLK1, H2AFX, CKS1B, BCCIP, NUSAP1, ANLN, CKS2, CENPF, CHAF1B, ACTG1, CDC6, PBK, CHEK1, ZWINT, ASPM, RAD51, MDC1, SMC4L1, TTK, MKI67, CDK2, MCM7, KIF15, MAP2K6, CDCA5, E2F1, BIRC5, UBE2C, BUB1, PKMYT1, ILF3, DLG7, DTYMK, MAD2L1, WEE1, SGOL1, CIT, KIF11, RBL1, MPHOSPH1, MDK, CCNA2, CDT1, PTTG1, CDC45L, FANCG, PLK4, MCM3, KNTC2, GMNN, HSPA2, FANCD2, CDK6, INHA, SKP2, SPAG5, UHRF1, CCNB1, CCNF, BUB1B, CCNB2, BRCA1, CDKN2C, AURKB, TPX2, CDC25A, PCNA, SMC2L1, IFITM1, CDK5RAP3, MCM2, CDC2, CDC25C, MCM6, CHAF1A, CDC20, CENPE
Spindle checkpoint	3	4.18E-03	TTK, CENPF, BUB1
Cytoskeleton organization and biogenesis	25	4.74E-06	CKS2, KIF2C, PRC1, KRT8, SPAG5, KIF14, KIF4A, DIAPH3, BUB1B, ESPL1, KIF23, KIF11, GTSE1, ZWINT, MPHOSPH1, STMN1, TTK, KIF20A, KIF15, KNTC2, NUSAP1, MGC39900, UBE2C, C9orf48, CENPE
Chromatin assembly or disassembly	9	8.89E-03	SUV39H1, HIST1H4C, CHAF1B, HMGB2, H2AFX, HIST1H2BH, CBX1, CENPA, CHAF1A
Nucleobase, nucleoside, nucleotide and nucleic acid metabolism	121	1.16E-08	SUV39H1, Pfs2, ADARB1, ATOH8, CDC7, TAF5, CITED4, TK1, HMG1L1, EXO1, PRIM1, LIG1, MCM5, TPD52L1, ATPIF1, PAPSS2, CBX1, RAD54L, POLE, ORC6L, EXOSC2, IQGAP3, RRM2, ORC1L, TRIP13, E2F2, POLQ, ITGB3BP, CENPA, NEIL3, CHTF18, RRM1, KNTC1, RNASEH2A, ID3, RFC5, FANCB, XRCC3, H2AFX, GATA2, HNRPA1, DMBX1, NASP, RFC4, RFC2, CENPF, CHAF1B, PAICS, POLD3, CDC6, SREBF1, CHEK1, GNE, MCM4, MXD3, RAD51, MDC1, UIP1, RAD51AP1, TIMELESS, FOXM1, EME1, SLBP, HIST1H4C, CDK2, HAT1, MCM7, NR3C1, DNMT1, BLM, RAD51C, E2F1, POLD1, FEN1, ILF3, SNRPA, DTYMK, RAB26, ZNF488, THOC4, RBL1, TYMS, RPA2, CDT1, PTTG1, CDC45L, FANCG, RPA3, HOXA2, GMNN, MCM3, RECQL4, FANCD2, POLA2, APEX2, FANCL, EZH2, NUDT1, PHF19, UHRF1, FOS, POLR3K, BRCA1, TOP2A, ASCC3L1, HIST1H2BH, POLE2, PCNA, NUDT21, HMGB2, RPA1, MCM2, MCM6, CHAF1A, PPIH, SLC2A4RG, TOPBP1, RFC3, TTF2, C20orf129, CSTF3
Second-messenger-mediated signaling	16	1.53E-05	APITD1, CKS2, RFC4, GABBR2, CCL2, HMGB2, HIST1H4C, TYMS, SPAG5, BUB1B, TOP2A, KNTC2, ZWINT, PCNA, UBE2C, FEN1
Meiosis	7	4.68E-04	CHEK1, NEK2, H2AFX, SGOL1, RAD54L, HSPA2, RAD51
Regulation of protein kinase activity	12	1.35E-04	CHEK1, CKS2, CDK5RAP3, TPD52L1, PKMYT1, BCCIP, CDC6, CDC25A, CDKN3, CCNA2, CDC25C, RGS4

Table 4 Pathway of down-regulated genes by troglitazone in SNU-668 cells

Term	Count	*P* value	Genes
DNA Polymerase	8	2.37E-06	PRIM1, RFC5, POLD3, POLE2, POLQ, POLD1, POLE, POLA2
Pyrimidine metabolism	13	6.71E-06	RRM2, TYMS, POLD3, TK1, DTYMK, RRM1, POLR3K, PRIM1, RFC5, POLE2, POLD1, POLE, POLA2
Purine metabolism	14	2.64E-04	RRM2, PAICS, POLD3, PDE7B, RRM1, POLR3K, PRIM1, RFC5, PDE4B, PAPSS2, POLE2, POLD1, POLE, POLA2
Cell cycle	34	3.15E-27	CDK6, BUB1, CDC7, SKP2, PKMYT1, CDC6, MAD2L1, CCNB1, BUB1B, ESPL1, CCNB2, CHEK1, WEE1, MCM5, MCM4, CDKN2C, CDC25A, PCNA, ORC6L, RBL1, ORC1L, MCM2, CDC2, CDK2, MCM6, CDC25C, CCNA2, CDK4, PTTG1, CDC45L, MCM7, PLK1, CDC20, MCM3

of p21-inducing cell-cycle-controlling genes, but also suppresses expression of genes associated with DNA composition and a variety of other genes. This suggests that transcription of many crucial genes is completely unrelated to PPAR-γ in the presence of troglitazone. As shown above, the growth-suppressive effects induced by ciglitazone and triglitazone occur *via* a PPAR-independent pathway, and transcription of a variety of genes associated with the induction of cell-cycle control and DNA compound factors are relevant to this process.

COMMENTS

Background

Peroxisome proliferation-activated receptor (PPAR)-γ is manifested in a variety

of tissues and cancer cells and the ligands that activate PPAR-γ are currently being studied as a novel treatment. The PPAR-γ ligand generally decreases the survival rate of cancer cells *via* differentiation, apoptotic induction, and changes in genes or proteins associated with entrance into the G1/S phase. We studied the appearance of PPAR-γ in two types of stomach cancer cells treated with ciglitazone and troglitazone, both of which induce PPAR-γ activation. We were able to identify cell proliferation, confirm the effects of PPAR-γ suppressors, and clarify any genetic alterations for the growth of stomach cancer cells using cDNA microarrays.

Research frontiers

They evaluated the effects of PPAR-γ and PPAR-γ agonists on stomach cancer cells at the cytological and molecular levels, and determined the concentration of troglitazone that can be used clinically to suppress the growth of stomach cancer cells. In 1999, Takahashi *et al* reported that stomach cancer is suppressed by PPAR-γ-ligand-mediated apoptosis. In 2005, Lu *et al* reported that troglitazone suppresses stomach cancer *via* the activation of PPAR-γ.

Innovations and breakthroughs

This manuscript shows a growth suppressing effect of the PPAR-γ ligands on stomach cancer cells *via* a pathway independent of PPAR-γ activation.

Applications

Currently, PPAR-γ is used to treat diabetes mellitus, hyperlipidemia, atherosclerosis, inflammatory vascular disease, Alzheimer's disease and some malignant diseases. In particular, the suppressing effect of the PPAR-γ ligands on stomach cancer cells may contribute to treatment efficacy.

Terminology

PPAR is a member of the family of nuclear receptors, which is part of the steroid/thyroid hormone/retinoid receptor superfamily. PPAR has three subtypes, α, β/δ, and γ. PPAR-γ has novel functions in cell division and differentiation, which are associated with inflammatory response, tissue resuscitation, vascular biology, and cancer formation, as a control factor for apoptosis.

Peer review

This manuscript describes a growth-suppressing effect of the PPAR-γ ligands on stomach cancer cell line SNU-668, but not SNU-216. This effect is independent of PPAR-γ activation. Associated with this, was an increase in p21 and decreased phosphorylated-ERK. The authors then went on to perform microarray analysis after treatment with PPAR-γ ligands.

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ORIGINAL ARTICLES

Therapeutic effects of four strains of probiotics on experimental colitis in mice

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Abstract

AIM: To investigate the therapeutic effects of four strains of probiotics (*E. feacalis*, *L. acidophilus*, *C. butyricum* and *B. adolescentis*) on dextran sulphate sodium (DSS)-induced experimental colitis in Balb/c mice.

METHODS: Eighty Balb/c mice were randomly divided into 8 groups. Weight-loss, fecal character, fecal occult blood and hematochezia were recorded daily. Disease activity index (DAI) scores were also evaluated everyday. Length of colon was measured and histological scores were evaluated on the 13th day. Myeloperoxidase (MPO) activity was detected. Interleukin-1 (IL-1) and IL-4 expression was detected by ELISA and RT-PCR.

RESULTS: The four strains of probiotics relieved the inflammatory condition of DSS-induced experimental colitis in mice. Weight loss was slowed down in all probiotics-treated mice. Even weight gain was observed by the end of probiotics treatment. The DAI and histological scores of probiotics-treated mice were lower than those of mice in the control group (1.9 ± 0.2 *vs* 8.6 ± 0.4, $P < 0.05$ for *E. faecalis*). The length of colon of probiotics-treated mice was longer than that of mice in the control group (10.3 ± 0.34 *vs* 8.65 ± 0.77, $P < 0.05$ for *E. faecalis*). The four strains of probiotics decreased the MP activity and the IL-1 expression, but increased the IL-4 expression. *E. faecalis* had a better effect on DSS-induced experimental colitis in mice than the other three strains.

CONCLUSION: The four strains of probiotics have beneficial effects on experimental colitis in mice. *E. faecalis* has a better effect on DSS-induced experimental colitis in mice than the other three strains. Supplement of probiotics provides a new therapy for UC.

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Key words: Probiotic; *E. faecalis*; Experimental colitis; Interleukin-1; Interleukin-4

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INTRODUCTION

Ulcerative colitis (UC) is a non-specific chronic inflammation of intestinal tract. The primary therapies with salazosulfamide, glucocorticoids and immunodepressant probiotics are limited by their side-effects, poor compliance of patients and high relapse rates[1-3].

It is well known that consumption of certain bacteria improves intestinal health[4-6]. Animal and clinical studies also indicate that gastrointestinal bacteria play an important role in the development of UC[7,8]. Supplements of probiotics provide a new therapy for UC. Bibiloni *et al*[9] found that VSL#3 (including 4 strains of *Lactobacilli*, 3 strains of *Bifidobacteria* and 1 strain of *Streptococcus*) displays a beneficial effect on UC.

Kruis *et al*[10] showed that *Escherichia coli* and Nissle 1917 have a similar effect and safety on 5-aminosalicylic acid[10]. It was reported that administration of *Lactobacillus casei* obviously decreases histological damage, while administration of *Escherichia coli* O83 and Nissle 1917 is beneficial for immunological regulation[11]. Peran *et al*[12] showed that probiotics can relieve UC symptoms through different mechanisms of action[12].

Because of the specific damage site of UC and the different colonizations of each bacterium, different probiotics have different effects on UC[13-15]. In order to find out effective strains, we compared the effects of four strains of probiotics on experimental colitis in mice.

MATERIALS AND METHODS

Bacterial strains

L. acidophilus, C. butyricum, B. adolescentis and *E. feacalis* were isolated from intestinal tract of healthy adults and identified in our facility.

Reagents

Salicylazosulfapyridine (SASP) was purchased from Fuda Pharmaceutical Co. Ltd (China). Dextran sulphate sodium was produced by MW 5000, Sigma-Aldrich Co (USA). ELISA kit was bought from Boster Biological Technology, Ltd (China), RT-PCR correlate agents were purchased from Promega Biotech Co., Ltd (USA) and Myeloperoxidase (MPO) diagnostic kit was bought from Jiancheng biotech Co (China).

Experimental design

Six-eight-week-old Balb/c mice (half males and half females, weighing 20.0 ± 2.0 g) provided by Hunan Agricultural University (China) were randomly divided into 8 groups, housed in clean filter-top cages under standard conditions (50% ± 10% humidity) in a 12-h dark/12-h light cycle, and fed with standard mouse chow. All mice were fed under standard conditions for 5 d. All mice were divided as follows: (1) Normal group: Normal diet without special treatment; (2) Model group: drinking 5% DSS for modeling; (3) NS group: 5% DSS for modeling + NS (100 μL/10 g) by gavage (4) SASP group: 5% DSS for modeling + SASP (50 mg/mL) by gavage; (5) *L. acidophilus* group: 5% DSS for modeling + 10^9 U/mL *L. acidophilus* by gavage; (6) *C. butyricum* group: 5% DSS for modeling +10^9 U/mL *C. butyricum* by gavage; (7) *B. adolescentis* group: 5% DSS for modeling +10^9 U/mL *B. adolescentis* by gavage; (8) *E. faecalis* group: 5% DSS for modeling +10^9 U/mL *E. faecalis* by gavage; The four strains of probiotics were grown overnight in culture media and suspended in normal saline (NS) to a concentration of 10^9 U/mL. SASP was dissolved in NS to a concentration of 50 mg/mL. The mice were fed with 200 μL of this daily-prepared suspension *via* an intragastric tube. On the 13th day, all mice were sacrificed.

To reflect the general conditions of mice, DAI scores were determined by an investigator blind to the protocol by scoring the extent of body weight loss, fecal character, fecal occult blood or hematochezia as previously described[16]. On the 13th day, blood was collected and all mice were sacrificed. The colon, from the colo-cecal junction to the anus was excised with its length measured, rinsed with 5 mL of 0.01 mol/L PBS (pH 7.4) to remove the fecal remnants, cut open longitudinally at the mesenteric attachment. One cm of the distal colon was removed and fixed for 48 h in PBS-buffered 10% formalin. The tissue was then processed for paraffin embedding and cut into 5-μm thick sections. The sections were stained with hematoxylin and eosin (HE). Other part of the colon was preserved in liquid nitrogen.

Table 1 PCR primers and products

	Primer		Product (bp)
IL-1β	Sense:	5'-AGCCCATCCTCTGTGACTCATG-3'	422
	Antisense:	5'-GCTGATGTACCAGTTGGGGAAC-3'	
IL-4	Sense:	5'-ACTTCAGTGGCTGGATTTAT-3'	424
	Antisense:	5' ATTCCCTGAAAGGCTTGGTC-3'	
β-actin	Sense:	5' ATGGATGACGATATCGCT-3'	569
	Antisense:	5'-ATGAGGTAGTCTGTCAGGT-3'	

MPO activity

MPO activity was measured as an indicator of neutrophil accumulation in colonic mucosa as preciously described[17].

RT-PCR

All primers were designed by software Primer5 (Table 1). The anneal temperature was 58-53℃ for 45 min at 30 amplification (Table 1).

ELISA analysis

The concentrations of IL-1 and IL-4 were measured in homogenized colons with an ELISA kit according to its manufacturers protocol and expressed as per milligram of protein.

Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 13.0. All results were expressed as mean ± SD. Data sets were analyzed by one-way analysis of variance (ANOVA) and Fishers' protected LSD post *hoc*-test. Those with a significant difference were further analyzed by Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

Weight loss

No difference was observed in body weight of mice among the 4 groups on day 0 (ANOVA). Body weight increased gradually in the normal group. Mice in the model group had a weight gain during the first 4 d due to lack of

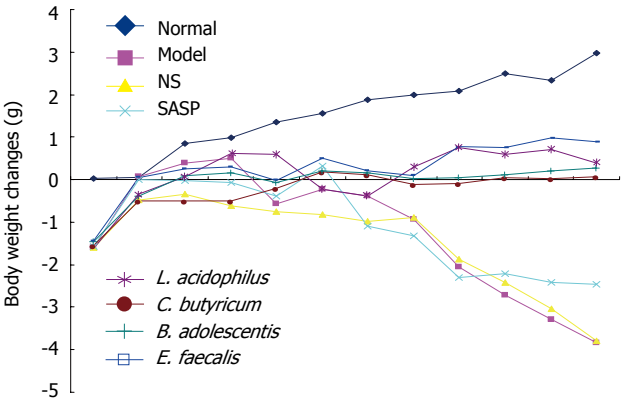

Figure 1 Body weight changes in all groups. The body weight of mice on the 1st day was taken as the basal level. The body weight of mice each day minus the basal body weight was expressed as the body weight change. The negative value indicates the decreased weight, and the positive value indicates the increased weight.

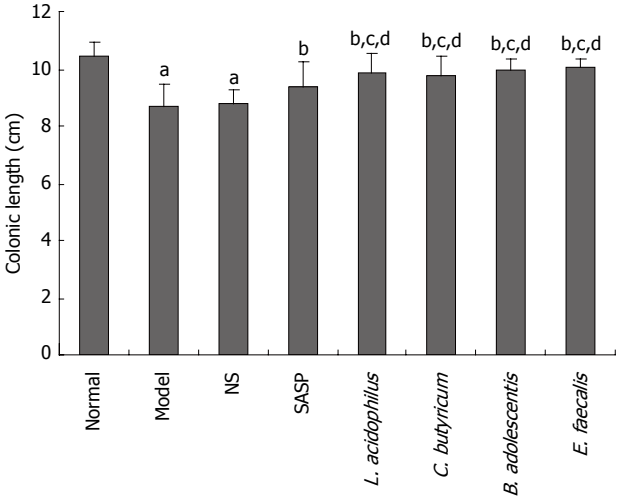

Figure 2 Length of colon on the 13th day. [a]$P < 0.05$ *vs* normal group; [b]$P < 0.05$ *vs* model group; [c]$P > 0.05$ *vs* normal group; [d]$P < 0.05$ *vs* SASP treatment group.

gavage stimulation, and a significant weight loss from day 5. Because of the double stimulation of DSS and gavages, mice in the NS group had an obviously weight loss. Mice in the SASP group maintained their body weight at its base level during the first 7 d, but had a significant weight loss from day 8 due to severe experimental colitis. The four strains of probiotics, especially *E. feacalis*, could inhibit the weight loss (Figure 1).

Length of colon

The length of colon of mice in the model and NS groups was significantly decreased compared with the SASP group. C. *butyricum*, L. *acidophilus*, *B. adolescentis*, *E. faecalis* could effectively prevent the shortening of colon (Figure 2).

DAI scores

Weight loss, fecal character, fecal occult blood and hematochezia were evaluated individually as previously described[16]. The highest DAI score was observed in

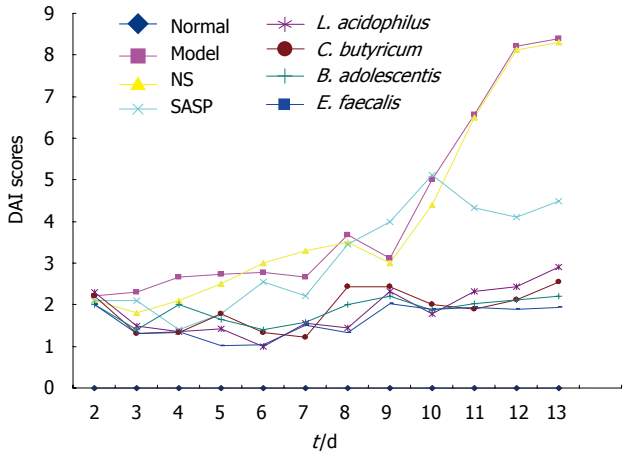

Figure 3 DAI scores of different groups. The DAI score was zero in normal group. The score increased gradually and reached 8.6 on the 13th day in model group. The score was low in SASP treatment group during the first 8 d, increased gradually during the last 5 d and reached 5.0 on the 13th day. The DAI scores of all four strains of probiotics were low in probiotics treatment groups, especially in *E. faecalis* treatment group. The maximum DAI score was only 1.9.

model and NS groups. SASP had a good effect on early experimental colitis, but a poor long-term effect on severe experimental colitis. The DAI scores of the four groups were low (Figure 3).

Histological scores

Histological changes in mice of the 4 groups were evaluated individually as previously described[18] (Figure 4). The highest score was found in mice of the model and NS groups, and a lower score was observed in mice of the SASP and probiotics treatment groups compared to mice of the model group, especially the mice in *E. faecalis* treatment group (1.67 ± 0.27 *vs* 9.99 ± 1.48, $P < 0.05$) (Figure 5).

MPO activity

The level of MPO activity was low in normal group, high in model group, and lower in SASP and probiotics treatment groups than in model group, especially in *E. faecalis* treatment group (Figure 6).

RT-PCR

The level of IL-1β mRNA was the lowest in normal group, the highest in model group, and lower in SASP and probiotics treatment groups than in model group (0.82 ± 0.03 *vs* 1.78 ± 0.07, $P < 0.05$) (Figure 7A-B). On the contrary, the level of IL-4 mRNA was the highest in normal group, the lowest in model group, higher in SASP and probiotics treatment groups than in model group (0.98 ± 0.01 *vs* 0.30 ± 0.01, $P < 0.05$) (Figure 8A-B)

ELISA analysis

The level of IL-1β and IL-4 was similar with that of mRNA. The level of IL-1β was the lowest *in L. acidophilus* and *E. faecalis* treatment groups (105.25 ± 7.79 *vs* 166.93 ± 13.69, $P < 0.05$), and the highest *in L. acidophilus*, *B. adolescentis*, and *E. faecalis* treatment groups (184.85 ± 11.51 *vs* 119.33 ± 10.86, $P < 0.05$) (Table 2).

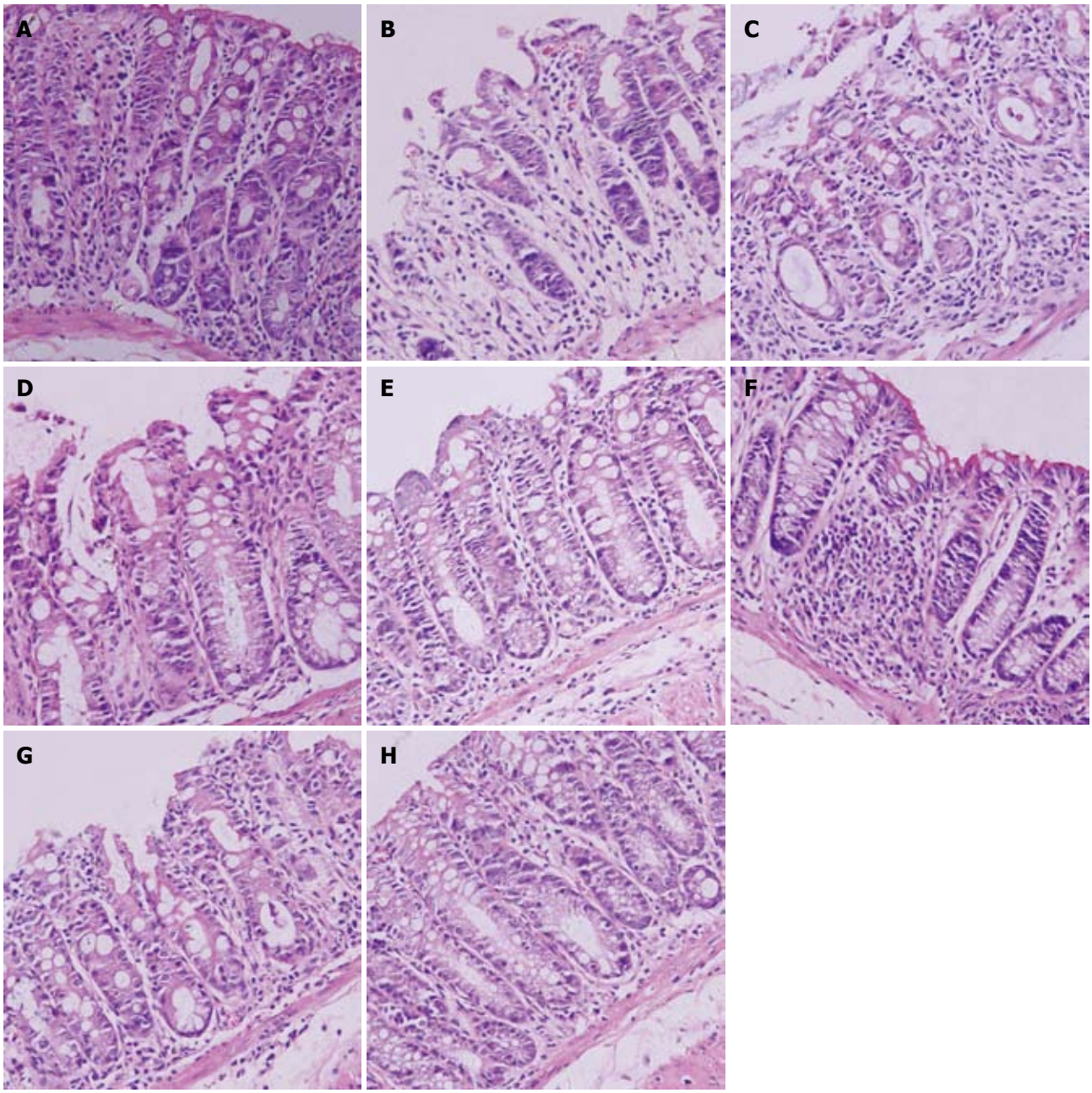

Figure 4 Histological images of mice. A: Normal group; B: Model group;C: NS group; D: SASP treatment group, E: *L. acidophilus* treatment group, F: *C. butyricum* treatment group, G: *B. adolescentis* treatment group, H: *E. faecalis* treatment group (HE, light microscope, x 200).

DISCUSSION

Studies showed that intestinal bacteria play an important role in the development of UC[5,19], and supplement of probiotic is beneficial for UC[13,14]. Because the damage site of UC resides mostly at the colon or rectum and different bacteria have different secretion functions or metabolism in intestinal tract, complement of local bacteria is more beneficial for UC. There are 300-500 different species of bacteria in the intestinal tract[5], and their location remains unclear at present. Since probiotics isolated from intestinal tract have an excellent permanent planting ability, obviously effective probiotics should be selected in a comparative study about their effects on experimental colitis.

Several studies on *Escherichia coli* Nissle, *Lactobacillus casei*, *Bifidobacterium lactis, Lactobacillus acidophilus, etc*, showed that these probiotics can be used in treatment of inflammatory bowel disease (IBD)[11,12,20]. In the present study, the effects of the four strains of probiotics on experimental colitis were compared.

The results of our study indicate that the four strains of probiotics had therapeutic effects on experimental colitis, confirming that probiotics can be used in treatment of colitis. Weight loss was slowed down and even weight gain was observed by the end of our experiment. The DAI and histological scores were low in probiotics treatment groups. These results agree with the reported findings[13,21]. The MPO activity decreased significantly in all probiotics treatment groups,

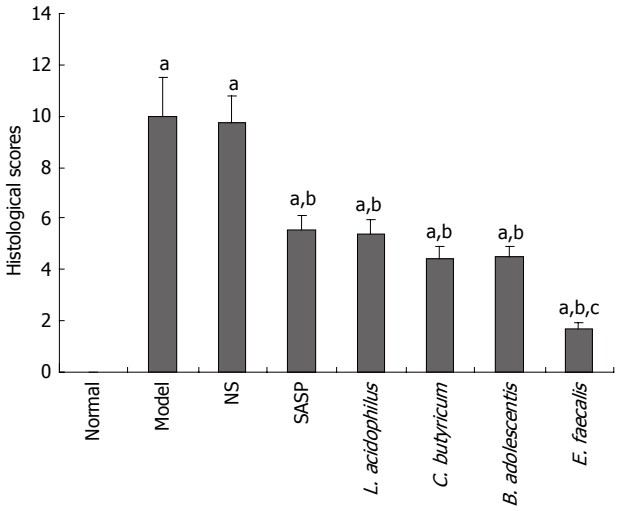

Figure 5 Histological scores of mice in different groups (mean ± SD, *n* = 10). The histological score was zero in normal group was zero ($^aP < 0.0$). The highest score was 9.9 ± 1.50 in model and NS groups ($^bP < 0.05$). The score was different in *E. faecalis* and SASP treatment groups ($^cP < 0.05$).

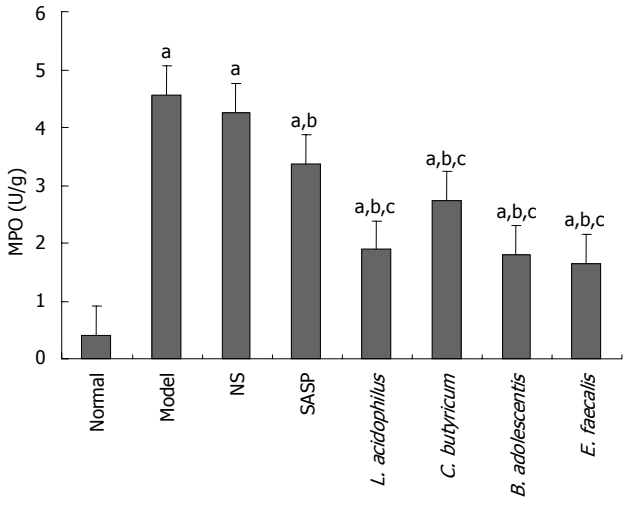

Figure 6 MPO activity in normal group (0.399 ± 0.133 U/g compared with normal group, model group and SASP treatment group. $^aP < 0.05$ *vs* normal group, $^bP < 0.05$ *vs* model group, $^cP < 0.05$ *vs* SASP treatment group.

Table 2 Concentrations of IL-1β and IL-4 used in ELISA analysis (mean ± SD)

Groups	Number	IL-1β (pg/mg.protein)	IL-4 (pg/mg.protein)
Norml	10	84.64 ± 7.02	205.81 ± 14.83
Model	10	166.93 ± 13.69[a]	119.33 ± 10.86[a]
NS	10	147.36 ± 12.61[a]	124.37 ± 9.85[a]
SASP	10	125.1 ± 12.04[a,c]	147.02 ± 12.02[a,c]
DSS + *L. acidophilus*	10	109.43 ± 11.98[a,c,e]	177.81 ± 10.29[a,c,e]
DSS + *C. butyricum*	10	121.25 ± 12.42[a,c,e]	150.76 ± 9.98[a,c]
DSS + *B. adolescentis*	10	120.27 ± 10.90[a,c]	173.69 ± 11.98[a,c,e]
DSS + *E. faecalis*	10	105.25 ± 7.79[a,c,e]	184.85 ± 11.51[c,e]

$^aP < 0.05$ *vs* normal group; $^cP < 0.05$ *vs* model group; $^eP < 0.05$ *vs* SASP group.

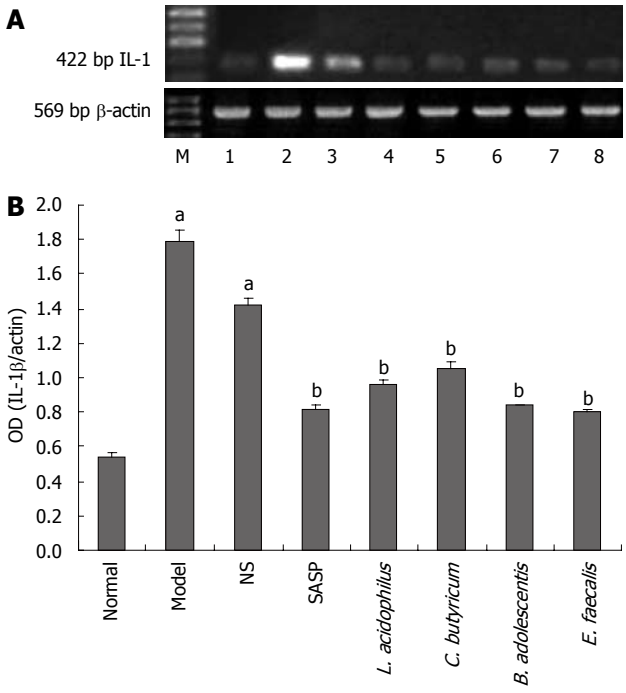

Figure 7 A-B Level of IL-1 βmRNA. M: Marker; 1: Normal group; 2: Model group; 3: NS group; 4: SASP treatment group; 5: *L. acidophilus* treatment group; 6: *C. butyricum* treatment group; 7: *B. adolescentis* treatment group; 8: *E. faecalis* treatment group. $^aP < 0.05$ *vs* normal group; $^bP < 0.05$ *vs* model group.

especially in *E. faecalis* treatment group, suggesting that the four stains of probiotics can relieve the symptoms of experimental colitis by decreasing the infiltration of neutrophils.

Balish *et al*[22] revealed that IBD occurs in germ-free IL-10-/- mice when they are colonized with a pure culture of *E. faecalis*, indicating *E. faecalis* is a conditioned pathogen. However, our study showed that *E. faecalis* could relieve the symptoms of experimental colitis and decrease the infiltration of neutrophils. *E. faecalis* had a better effect on experimental colitis than the other three strains of probiotics. Ruiz *et al*[23] reported that the expression of pro-inflammatory cells is transient 1 wk after *E. faecalis* treatment in intestinal epithelial cells from wild-type mice, suggesting that lack of protective TGF-β/Smad signaling and failing to inhibit TLR2-mediated pro-inflammatory gene expression in the intestinal epithelium might be a partial mechanism of IBD developed in IL-10-/- mice.

Because *E. faecalis*, *L. acidophilus* and some other probiotics have different effects on experimental colitis in wild-type mice rather than in immunodeficient mice, there should be more immunological mechanisms against experimental colitis except for the signal pathway or innate immunological mechanism. Some inflammatory cytokines, especially IL-1 and IL-4, are closely related with the development and progress of experimental colitis and IBD. IL-1 is secreted by mononuclear macrophages and high doses of IL-1, especially IL-1β, can result in UC[24]. IL-4 is synthesized by lamina propria intestinal lymphocytes after *in vitro* polyclonal activation. Compared with peripheral lymphocytes, intestinal epithelial and lamina propria lymphocytes spontaneously secrete IL-4[25], which can inhibit secretion of IL-1β by monocytes in a dose-dependent manner[26], suggesting

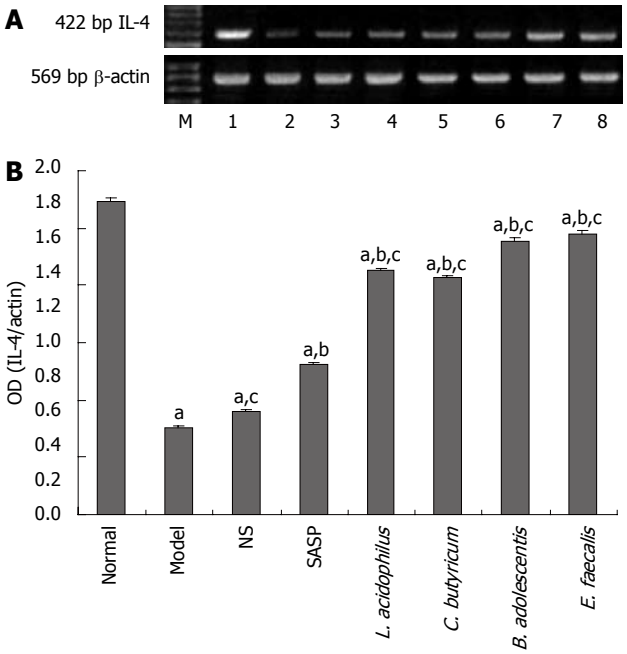

Figure 8 A-B Level of IL-4 mRNA. M: Marker; 1: Normal group; 2: Model group; 3: NS group; 4: SASP treatment group; 5: *L. acidophilus* treatment group; 6: *C. butyricum* treatment group; 7: *B. adolescentis* treatment group; 8: *E. faecalis* treatment group. $^{a}P < 0.05$ *vs* normal group; $^{b}P < 0.05$ *vs* model group; $^{c}P < 0.05$ *vs* SASP treatment group.

that IL-4 has a protective function on regulatiing immunological reaction in intestinal tract. Our study showed that the expression of IL-1β was lower in probiotics treatment groups than in model group, and similar to that in SASP treatment group. The expression of IL-4 in probiotics treatment groups was increased. The expression of IL-4 was higher in *E. feacalis* treatment group than in treatment SASP group. The general condition, DAI scores, histological scores, and MPO activity were maintained at a parallel level, supporting the effects of probiotics at cytokine level. Changes in IL-1 and IL-4 level represent the inflammation degree of experimental colitis. Our experimental results also indicate that there should be some other inflammatory cytokines involved in the difference of adaptive immunological mechanisms in experimental colitis of wide-type and immunodeficient mice.

In summary, *L. acidophilus, C. butyricum, B. adolescentis, E. faecalis* are effective against DSS-induced acute experimental colitis. Reduced infiltration of neutrophils, decreased expression of IL-1 and increased expression of IL-4 might be a partial immunological mechanism of probiotics on experimental colitis in mice.

COMMENTS

Background

Ulcerative colitis (UC) is a non-specific chronic inflammation of intestinal tract and the primary therapies with probiotics are limited by their side-effects, poor compliance of patients and high relapse rates. Supplement of probiotics provides a new therapy for UC.

Research frontiers

Bacteria play an important role in pathogenesis of UC. Supplement of probiotics provide a new therapy for UC. Because of the specific damage site of UC and the different colonization sites of bacteria, different probiotics display different effects on UC. A more effective strain of probiotics should be selected for UC.

Innovations and breakthroughs

They compared the effects of four strains of probiotics isolated from healthy human feces in order to find one or two more effective strains. *E. faecalis* had a better effect than the other three strains. Their study showed that *E. faecalis* had different effects on experimental colitis in wild-type mice. The experimental results indicate that there should be some other inflammatory cytokines involved in experimental colitis of wide-type and immunodeficient mice except for IL-1 and IL-4.

Applications

The results of their study indicate that there should be some other inflammatory cytokines involved in experimental colitis of wide-type and immunodeficient mice except for IL-1 and IL-4.

Peer review

The effects of four strains of probiotics on DSS-induced colonic inflammation in mice were clarified. The authors showed that the four strains of probiotics derived from healthy human feces could relieve colonic inflammation as sulfasalazine. Of the four strains, *E. faecalis* was most beneficial for DSS-induced colitis. Methods employed and results obtained are reasonable.

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BRIEF ARTICLES

Evolution and predictive factors of thyroid disorder due to interferon alpha in the treatment of hepatitis C

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Abstract

AIM: To study predictive factors of thyroid dysfunction associated with interferon-alpha (IFNα) therapy in chronic hepatitis C (CHC) and to describe its long-term evolution in a large population without previous thyroid dysfunction.

METHODS: We performed a follow-up of thyroid function and detection of thyroid antibodies in 301 patients treated for CHC with IFNα from 1999 to 2004.

RESULTS: Thyroid disorder developed in 30/301 (10%) patients with a mean delay of 6 ± 3.75 mo: 13 patients had hyperthyroidism, 11 had hypothyroidism, and 6 had biphasic evolution. During a mean follow-up of 41.59 ± 15.39 mo, 9 patients with hyperthyroidism, 3 with hypothyroidism, and 4 with biphasic evolution normalized thyroid function in 7.88 ± 5.46 mo. Recovery rate of dysthyroidism was not modified by treatment discontinuation, but was better for patients with negative thyroid antibodies before antiviral treatment (P = 0.02). Women had significantly more dysthyroidism (P = 0.05). Positive thyroid peroxidase and thyroglobulin antibodies were more frequent before antiviral treatment in patients who developed dysthyroidism (P < 0.0003 and P = 0.0003, respectively). In a multivariate model, low fibrosis was found to be a predictive factor of dysthyroidism (P = 0.039).

CONCLUSION: In this monocentric population of CHC, dysthyroidism, especially hyperthyroidism, developed in 10% of patients. Low fibrosis was found to be a predictive factor of dysthyroidism. Thyroid disorder recovered in 16/30 patients (53%) and recovery was better in the non-autoimmune form.

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Key words: Chronic hepatitis C; Interferon alpha; Predictive factors; Thyroid disorder

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INTRODUCTION

Three alpha interferons and two peg-interferons are currently commercially available for the treatment of chronic hepatitis C (CHC). Since 2001, peg-interferons have been used in association with ribavirin[1,2], and they have become the reference treatment for CHC since the French consensus conference in 2002[3]. In the presence or absence of ribavirin, interferon-alpha (IFNα) has a well-known side effect profile. Some side effects are common, such as pseudo-flu syndrome, headaches, myalgia, fever, wasting, leucopoenia or thrombocytopoenia. Indeed, clinicians often reduce the dose or sometimes discontinue IFNα in those patients who develop thyroid dysfunction, thus possibly compromising the therapeutic response to this treatment. In 1995, Preziati *et al*[4] reported that 9.3% of patients with CHC receiving IFNα developed thyroid dysfunction. During the treatment of CHC, IFNα-induced thyroid dysfunction appears in 3% to 15% of cases[5-9], with various clinical presentations. In previous

studies, the number of patients included was insufficient in certain cases[4,5,10,11], and other studies did not exclude patients with a past history of dysthyroidism[4,8,9,12,13]. However, the long-term course and the risk factors of thyroid disorder are not well understood[6,7,14].

In this single-center study we report on a large population of patients without previous thyroid dysfunction who underwent IFNα treatment for CHC. Our objectives were to describe the prevalence and long-term course of thyroid disorder in this population and to assess the factors that are predictive of dysthyroidism.

MATERIALS AND METHODS

Patients

We studied all patients with CHC, treated with IFNα from January, 1999 to May, 2004 at the Department of Hepatology and Gastroenterology at Bicêtre Hospital (Kremlin-Bicêtre, France). Patients with human immunodeficiency virus or hepatitis B virus co-infection, hemophiliacs and patients with a past history of thyroid disorder were systematically excluded. Before 2002, a liver biopsy was performed in each patient in order to evaluate inflammatory activity and the stage of liver fibrosis measured by the Metavir score[15]. Since 2002, liver biopsy was performed only in patients with genotype 1, 4 or 5. Patients received standard interferon alpha (2a), 3 MU subcutaneously thrice weekly, peg-interferon alpha (2b) (Viraferon peg®, Schering Plough, NJ, USA) 1.5 μg per kilogram of body weight subcutaneously once weekly or peg-interferon alpha (2a) (Pegasys®, Hoffmann-La Roche, Ltd, Switzerland) 180 μg subcutaneously once weekly, with or without 800 mg to 1200 mg of ribavirin per day. Thyroid-stimulating hormone (TSH) was measured before and every eight weeks during the antiviral treatment. Therapeutic follow-up of thyroid disorder was then performed until June, 2006. Thyroid peroxidase antibodies (TPOAb), thyroglobulin antibodies (TgAb) and thyroid-stimulating hormone receptor antibodies (TSHRAb) were measured before the start of antiviral treatment and after the diagnosis of thyroid disorder as necessary.

Methods

The diagnosis of CHC was based on positive hepatitis C virus antibodies assessed by a second or third generation enzyme immunoassay. Hepatitis C virus RNA was measured by polymerase chain reaction amplification of viral RNA from serum. Viral genotypes were determined using a hybridization technique (INNO-LIPA HCV, Innogenetics, Gent, Belgium). The serum levels of TSH were measured using the AutoDELFIA™ TSH Ultra assay (sensitivity 0.03 MIU/L, total analytical variation < 5%) from Wallacoy, Turku, Finland. The normal TSH range in our laboratory was 0.3-4.0 MIU/L. The serum levels of TPOAb, TgAb and TSHRAb were measured using radioimmunoassays or radioimmunometric assays. The normal ranges in our laboratory were TPOAb < 60 IU/L, TgAb < 60 IU/L and TSHRAb < 5 IU/L.

Thyroid dysfunction was diagnosed when TSH level was either < 0.3 MIU/L (hyperthyroidism) or > 4.0 MIU/L (hypothyroidism) by two successive tests. Thyroid ultrasonography or thyroid scintigraphy was performed according to the clinical judgement of the endocrinologist. We have previously found three profiles of dysthyroidism: hyperthyroidism, hypothyroidism and a short hyper-followed by long hypothyroidism classically named biphasic evolution[9,14,16].

Table 1 Characteristics of the study population

Characteristics	Total group (n = 301)
Age[1] (yr)	48.27 ± 11.79
Gender (%, M/F)	57.5/42.5
Contamination mode (%)	
Tranfusion	27.2
Drug-addict	26.9
Blood exposure accident	7.6
Others	38.2
Genotype (% with 1/2/3/4/5/ND)	35/11/18/8/2/26
Stage of fibrosis (% with stage 0/1/2/3/4/ND)	1/11/42/20/20/6
Type of treatment (%)	
With IFNα	3.3
Peg-interferon	9.0
IFNα + ribavirin	27.2
Peg-interferon + ribavirin	60.4
Duration of treatment (mo)[1]	7.91 ± 3.78
TSH before treatment (MIU/L)[1]	1.54 ± 1.25
Positive antibodies before treatment	
TPOAb	12/229
TGAb	8/227
TSHRAb	1/95

ND: Not determined. [1]mean ± SD.

Statistical analysis

The predictive values of the following factors were analyzed: patients age at onset of the antiviral treatment, gender, mode of contamination, viral load and genotype, grade of histological fibrosis, type and duration of the antiviral therapy, TSH levels and the presence of TgAb, TPOAb or TSHRAb before the antiviral treatment.

Descriptive statistics were obtained using the Kruskal-Wallis test as appropriate, followed by a multivariate logistic regression analysis. A two-tailed P value < 0.05 was considered significant. Data analysis was performed using the EPI-Info Statistical Package (version 3.2.2).

RESULTS

Characteristics of the study population

The main characteristics of the 301 studied patients are shown in Table 1. Genotype status was known in 224 (74%) of 301 patients, as it was not known for the patients treated before 2002. The stage of fibrosis based on the Metavir score was obtained for 94% of patients. Patients with genotype 2 or 3 treated after 2002 did not have systematic evaluation of fibrosis before antiviral treatment. 247 (87%) of the patients who had a biopsy, had moderate or severe fibrosis (equal to or more than F2). In the inclusion period, from 1999 to 2004, there was heterogeneity in the antiviral treatment. However, the majority (60.4%) of the study population received

Table 2 Classification of thyroid disorder and long-term normalisation of dysthyroidism (n = 30)

	Type of dysthyroidism of normalisation	Discontinued treatment	Normalisation	Mean delay (mo)
Hyperthyroidism	13	9	9	7.44 ± 7.05[1]
Silent thyroiditis	5	4	5	6.80 ± 3.70[1]
Graves' disease	4	2	1	5
NC	2	2	2	2.00 ± 1.41[1]
Triphasic evolution	2	1	1	24
Hypothyroidism	11	6	3	10.67 ± 4.04[1]
Autoimmune	10	5	2	8.50 ± 2.12[1]
NC	1	1	1	15
Biphasic evolution	6	3	4	10.75 ± 5.50[1]

NC: Not classified. [1]mean ± SD.

peg-interferon alpha and ribavirin bitherapy. The TSH level before the antiviral treatment was known for all studied patients and was within normal ranges.

Prevalence of thyroid dysfunction during the antiviral treatment

Amongst the 301 patients with CHC, 30 (10%) developed biochemical thyroid dysfunction (TSH < 0.3 or > 4.0 MIU/L) during the antiviral treatment, 17 women and 13 men. Hyperthyroidism was seen in 13 (43%) of the 30 cases, hypothyroidism in 11 (37%) and biphasic evolution in 6 (20%). Table 2 shows the prevalence and classification of the thyroid disorders. The investigative work-up performed for each case with thyroid dysfunction, classified 11 of the 13 patients with hyperthyroidism as Graves' disease, silent thyroiditis or triphasic evolution, and 10 of the 11 patients with hypothyroidism as autoimmune hypothyroidism. Graves' disease was defined by the presence of clinical hyperthyroidism with positive TSHRAb and diffusely increased radioactive iodine intake on thyroid scintigraphy. Silent thyroiditis was defined by thyrotoxicosis, no tender goitre, and markedly decreased radioactive iodine intake on thyroid scintigraphy. Cases of "triphasic evolution" were defined as reported by Bohbot *et al* in 2006[17] (unusual evolution of silent thyroiditis to Graves' disease). Autoimmune hypothyroidism was defined by positive TgAb and/or TPOAb associated with clinical hypothyroidism.

Long-term course of dysthyroidism

Dysthyroidism occurred at an average of 6 ± 3.75 mo after the beginning of antiviral treatment. The evolution of the different profiles of dysthyroidism was described (Table 2) during a long-term follow-up (41.59 ± 15.35 mo) after the diagnosis of dysthyroidism. We observed that practitioners did not have the same attitude with respect to the evolution of dysthyroidism because the antiviral treatment was more frequently discontinued in hyperthyroidism (69%) than in hypothyroidism (55%) or biphasic evolution (50%). Four patients with hyperthyroidism did not require discontinuation of antiviral treatment. Among them, 1 patient presented with transient Graves' disease and another died before the end of follow up, 1 patient continued the antiviral treatment for only one month because hyperthyroidism occurred at the end of antiviral treatment and the type of hyperthyroidism could not be classified in 1 patient. Hypothyroidism that needed discontinuation of antiviral treatment was more frequently autoimmune hypothyroidism with TSH > 50 MIU/L except in 3 patients (1 with not classified with hypothyroidism and 2 with moderate elevation of TSH). Concerning therapeutic normalisation, no difference was observed regarding the discontinuation of antiviral treatment (56.6% *vs* 50.0%, NS). Treatment for thyroid disease was administered to 14 symptomatic patients (5 patients received carbimazole and 9 levothyroxine).

Prevalence of positive thyroid antibodies before antiviral treatment

Amongst the patients tested for TPOAb (n = 229), TgAb (n = 227) and TSHRAb (n = 94) before antiviral treatment, 12 (5%) were found to be positive for TPOAb (> 60 IU/L), 8 (3%) positive for TgAb (> 60 IU/L), and only 1 (1%) for TSHRAb (>5 IU/L). None of these patients had thyroid disorder before the introduction of IFNα. 7/12 patients with positive TPOAb and 4/8 patients with positive TgAb developed thyroid disorder during the antiviral treatment. The patients who had positive pretherapeutic TSHRAb did not develop Graves' disease. Regarding the presence of autoantibodies, IFNα induced-thyroid disease was classified as "autoimmune form" and "non-autoimmune form" similar to Mandac *et al*[18]. The autoimmune form was defined by the development of thyroid antibodies with or without clinical disease, including both autoimmune hypothyroidism and Graves' disease. The non-autoimmune form was defined by destructive thyroiditis or hypothyroidism with negative thyroid antibodies. We observed that patient recovery was significantly better in the non-autoimmune form than in the autoimmune form (33.3% *vs* 66.7%, P = 0.02).

Prediction of thyroid dysfunction

As shown in Table 3, we initially performed a univariate analysis using eight covariates (age, gender, contamination mode, genotype, stage of histological fibrosis, type of antiviral treatment [monotherapy with standard IFNα or peg-interferon versus combination of standard IFNα or peg-interferon with ribavirin] and duration, positive autoantibodies before the antiviral

Table 3 Features associated with dysthyroidism

	With dysthyroidism	Without dysthyroidism	*P*
Age[1] (yr)	46.20 ± 10.08	48.49 ± 11.96	0.40
Female (%)	56.9	40.9	0.05
Contamination mode (%)			0.33
Transfusion	33.3	26.6	
Drug-addict	20.0	27.7	
Blood exposure accident	13.3	7.0	
Others	33.3	38.7	
Genotype (%, 1/2/3/4/5/6/ND)	57/13/13/0/0/0/17	33/11/20/9/1/0/26	0.20
Stage of fibrosis < F2 (%)	30.0	10.3	0.009
Type of treatment (%)		0.47	
IFNα	3.3	3.3	
Peg-interferon	6.7	9.3	
IFNα + ribavirin	16.7	28.5	
Peg-interferon + ribavirin	73.3	58.9	
Duration of treatment[1] (mo)	7.73 ± 3.64	8.09 ± 3.93	0.30
Positive antibodies before treatment (%)			
TPOAb	26.9	2.5	< 0.0003
TgAb	18.5	1.5	0.0003
TSHRAb	0.0	1.3	0.42

ND: Not determined. [1]mean ± SD.

treatment: TPOAb, TgAb and TSHRAb). Four covariates were associated with dysthyroidism (gender, stage of histological fibrosis, positive TPOAb and TgAb). Secondly, in a multivariate logistic regression analysis of predictive factors of dysthyroidism using those four covariates, one predictive factor was found. The index of fibrosis was significantly less for patients with dysthyroidism than for patients without dysthyroidism. The stage of fibrosis was less than 2 units (mild fibrosis) in 30.0% of patients with dysthyroidism *vs* 10.3% of patients without dysthyroidism (OR, 0.56; 95% IC, 0.33-0.97; $P = 0.039$). There was a non significant trend towards positive TPOAb before antiviral treatment for patients with dysthyroidism. Amongst patients with positive TPOAb before antiviral treatment, 7 (26.9%) developed dysthyroidism *vs* 5 (2.5%) who did not (OR, 5.31; 95% IC, 0.80-35.16; $P = 0.083$).

DISCUSSION

The prevalence of thyroid dysfunction during IFNα therapy for CHC was 10% in our series. Amongst the 301 patients with CHC, hyperthyroidism was more frequent (13/30) than hypothyroidism or biphasic evolution. The mean follow-up of thyroid disorder in our study was 41.59 ± 15.39 mo, 53% of patients recovered from thyroid disease without a difference regarding the discontinuation of antiviral treatment. Mild fibrosis was found to be an independent predictive factor of dysthyroidism during antiviral treatment.

Our single center study included a large population of 301 patients with CHC and we performed a long term follow-up of these patients, not only during the antiviral treatment, but also after treatment, to detect dysthyroidism in patients who had no previous thyroid dysfunction. Although the patient data were retrospective, the follow-up data were partly prospective. This may explain some of the heterogeneity in the type of antiviral treatment used. In addition, the conditions under which the antiviral treatment was stopped when dysthyroidism developed were not well defined. We evaluated the presence of positive thyroid antibodies using the same methods in all patients, and an investigative work-up of the pathology was performed for each case of thyroid dysfunction.

The prevalence of hyperthyroidism found in our study (43% *vs* 37% hypothyroidism) is unusual. Previous studies have reported more hypothyroidism (two out of the three cases) than hyperthyroidism (one out of the three cases)[19] with the exception of Benelhadj *et al*[5] and Hsieh *et al*[11]. Hsieh *et al*[11] explained this difference as being related to the population's eating habits, yet our study population was not particularly exposed to an increased risk of dysthyroidism due to eating habits. Benelhadj *et al*[5] did not explain this difference as only 6 patients developed thyroid dysfunction. The discrepancy may be partly explained by the findings of several other studies including silent thyroiditis developing into hypothyroidism or biphasic evolution whereas this disease usually begins with hyperthyroidism. Furthermore, in our series, hyperthyroidism cases included 30% (4/13) with Graves' disease, which is in the same range as a previously published series[20].

In accordance with the presence of at least one thyroid antibody, we classified thyroid disorder, as autoimmune and non-autoimmune, which seemed to be predictive of the evolution of dysthyroidism. In this study we should have based the autoimmune form on at least one positive thyroid antibody rather than consider each positive antibody separately. Three of the four cases of Graves' disease developed following IFNα therapy and did not recover after the end of the antiviral therapy. This suggests that IFNα triggered the development of Graves' disease in predisposed individuals[20]. In silent thyroiditis, which is a non-autoimmune IFNα-induced thyroiditis, four patients recovered without

the addition of specific treatment when interferon was discontinued and one recovered without discontinuing antiviral treatment. This suggests that the autoimmune mechanism is more deleterious in IFNα-induced thyroid disease. Among the eleven hypothyroidism patients, therapeutic normalisation was obtained in 3 (27%) within 10.67 ± 4.04 mo. Also, the patients who developed autoimmune forms of hypothyroidism, such as autoimmune hypothyroidism, did not recover after cessation of IFNα treatment and systematically needed T4 replacement during the follow-up.

In the multivariate analysis, one factor was significantly correlated with the development of dysthyroidism during antiviral treatment: the stage of fibrosis below the F2 Metavir score. However, patients treated with IFNα had more severe fibrosis (82% of patients with a stage of fibrosis equal to or above F2). Perhaps this was correlated to the variability in the autoimmune response to hepatitis C virus infection, however, this predictive factor will require further study. Surprisingly, the presence of TPOAb before the introduction of antiviral treatment was not significant in the multivariate model whereas it was in the univariate analysis; this may have been due to the small number of patients with positive antibodies. Kabbaj *et al*[21], found three predictive factors for dysthyroidism in a univariate analysis: female gender, positive anti TPO antibodies before antiviral treatment and TSH before antiviral treatment (even if it was still in the normal ranges). We do not understand why the variable "stage of fibrosis under F2" is mentioned in the statistical analysis because only patients with fibrosis equal or more than F2 were treated. Kee *et al*[22], found that only female gender was predictive of dysthyroidism in a multivariate model. Thyroid microsomal antibody was found to be predictive of thyroid disease in a case-control study. There were no significant differences between thyroid dysfunction patients in the case-control study with respect to liver inflammation and fibrosis grade, however, the authors used the Knodell score which does not distinguish activity and fibrosis.

Some practical guidelines may be drawn from this study: the TPOAb state should be determined in patients before introducing IFNα and a regular follow-up of TSH every two mo or less is needed in patients with a risk of dysthyroidism (low fibrosis, female gender, positive TPOAb). Finally, two distinct mechanisms are described in the development of thyroid disorder during IFNα therapy: autoimmune and non-autoimmune-induced thyroid dysfunction. With regard to our results, the autoimmune form seems to have more severe consequences and longer evolution, which indicates the importance of early detection, in order to adapt the follow-up of thyroid function and therapy without discontinuing the antiviral treatment, since the discontinuation of antiviral treatment seems to have no predictive value on the evolution of dysthyroidism.

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COMMENTS

Background

Alpha interferons and peg-interferons have successively become the reference treatment for chronic hepatitis C with or without ribavirin. They have both induced thyroid dysfunction in 3% to 15% of cases. Indeed, clinicians often reduce the dose or sometimes discontinue interferon-alpha (IFNα) in patients who develop thyroid dysfunction, thus possibly compromising the therapeutic response to this treatment. In previous studies, the number of patients included was insufficient in certain cases, and other studies did not exclude patients with a past history of dysthyroidism. However, the long-term course and the risk factors for thyroid disorder are not well understood.

Research frontiers

Despite the role of IFNα, the pathogenesis of thyroid disease remains uncertain; also it seems to be related to an immunologic predisposition. Therefore, the authors tried to determine the risk factors which influence thyroid dysfunction.

Innovations and breakthroughs

Two distinct mechanisms are described for the development of thyroid disorder during IFNα therapy: autoimmune and non-autoimmune- induced thyroid dysfunction. With regard to our results, the autoimmune form seems to have more severe consequences and longer evolution, which indicates the importance of early detection, in order to adapt the follow-up of thyroid function and therapy without discontinuing the antiviral treatment, since the discontinuation of antiviral treatment seems to have no predictive value on the evolution of dysthyroidism. Furthermore, the stage of fibrosis below the F2 Metavir score was significantly correlated with the development of dysthyroidism during antiviral treatment. We hypothesized that low fibrosis, associated with better HCV response, was also associated with autoimmune activation, including the development of anti-thyroid autoantibodies.

Applications

Some practical guidelines may be drawn from this study: the TPOAb state in patients should be determined before introducing IFNα and a regular follow-up of TSH every two mo or less is needed in patients with a risk of dysthyroidism (low fibrosis, female gender, positive TPOAb).

Peer review

This is a fairly good written manuscript. But the authors need to deal with the issue of predictive factors for developing dysthyroidism in detail in the discussion section and make a plausible explanation about the difference from the previous papers.

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BRIEF ARTICLES

Prevalence of bile reflux in gastroesophageal reflux disease patients not responsive to proton pump inhibitors

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Abstract

AIM: To determine the prevalence and characteristics of bile reflux in gastroesophageal reflux disease (GERD) patients with persistent symptoms who are non-responsive to medical therapy.

METHODS: Sixty-five patients (40 male, 25 female; mean age, 50 ± 7.8 years) who continued to report symptoms after 8 wk of high-dose proton pump inhibitor (PPI) therapy, as well as 18 patients with Barrett's esophagus, were studied. All patients filled out symptom questionnaires and underwent endoscopy, manometry and combined pH-metry and bilimetry.

RESULTS: There were 4 groups of patients: 22 (26.5%) without esophagitis, 24 (28.9%) grade A-B esophagitis, 19 (22.8%) grade C-D and 18 (21.6%) Barrett's esophagus. Heartburn was present in 71 patients (85.5%) and regurgitation in 55 (66.2%), with 44 (53%) reporting simultaneous heartburn and regurgitation. The prevalence of pathologic acid reflux in the groups without esophagitis and with grades A-B and C-D esophagitis was 45.4%, 66.6% and 73.6%, respectively. The prevalence of pathologic bilirubin exposure in these 3 groups was 53.3%, 75% and 78.9%, respectively. The overall prevalence of bile reflux in non-responsive patients was 68.7%. Pathologic acid and bile reflux was observed in 22.7% and 58.1% of non-esophagitic patients and esophagitic patients, respectively.

CONCLUSION: The high percentage of patients poorly responsive to PPI therapy may result from poor control of duodenogastroesophageal reflux. Many patients without esophagitis have simultaneous acid and bile reflux, which increases with increasing esophagitis grade.

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Key words: Gastroesophageal reflux disease; Duodenogastric reflux; Bile reflux; Bilirubin; Barrett's esophagus

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INTRODUCTION

As a result of their strong acid suppression, proton pump inhibitors (PPIs) have been used to treat most patients with gastroesophageal reflux disease (GERD)[1-5]. Acid reflux is the main risk factor for GERD, with pH-metry being the standard method used in the diagnosis of GERD. Many patients with typical GERD symptoms, however, have been found to have a negative pH-metry[6]; these patients have been found to differ in symptoms, response to medical therapy, and endoscopy results from patients with positive pH-metry.

Although the role of acid reflux in GERD has been established, and links between acid and bile reflux have been found, less is known about the role of bile in the pathogenesis of esophageal mucosal damage. Thus, the incidence of GERD, its clinical impact, etiology, evolution and therapeutic implications cannot be determined directly. This limitation, however, was improved by the introduction of bilimetry in clinical practice[7]. This method uses spectrophotometric analysis to measure the

presence of bilirubin in the refluxate, thus providing a direct and reliable measurement of bile reflux.

The combination of pH-metry and bilimetry has increased the sensitivity and accuracy of GERD diagnosis and has shown that increased bile reflux is correlated with increased severity of esophagitis[8-9]. Moreover, other authors showed that a high percentage of GERD patients, poorly responsive to PPI therapy, had mixed acid and bile reflux, or isolated bile reflux[10]. To expand these investigations, we evaluated the prevalence and characteristics of bile reflux in GERD patients with persistent symptoms who were non-responsive to medical therapy.

MATERIALS AND METHODS

Patients

Of 230 patients with heartburn and regurgitation evaluated between January, 2002 and July, 2006, 65 (40 male, 25 female; mean age, 50 ± 7.8 years) continued to report symptoms after 8 wk of high-dose PPI therapy (40 mg esomeprazole *bid*). In addition, 18 patients with Barrett's esophagus were included. All patients were administered symptom questionnaires and underwent endoscopy, perfused esophageal manometry and combined 24-h esophago-gastric pH-bilimetry.

Endoscopy (EGDS)

The presence of esophagitis was classified according to the Los Angeles Classification[11]. The presence of hiatal hernia was determined and esophageal biopsies were used to diagnose Barrett's esophagus.

Perfused esophageal manometry

Manometric evaluation was made without sedation after 1 wk of pharmacologic wash-out and an overnight fast. An 8-channel manometric device (Menfis Biomedical Inc. Bologne, Italy) connected to a low compliance hydro-pump (Arndorfer Medical Specialties, Greendale, Wisconsin, USA) was used. The 8 open tip (4 radial and 4 longitudinal) manometric probe was inserted through the nose into the stomach and lower esophageal sphincter (LES) parameters (pressure, length and postdeglutitive relaxation) were evaluated by a rapid and stationary pull-through technique. Esophageal motor activity (amplitude and duration of waves, percentage of peristaltic and simultaneous post-deglutitive sequences) was evaluated with stationary pull-through after 20 wet and dry swallows.

Twenty-four hours esophago-gastric pH-metry

We performed this test after 1 wk of pharmacologic wash-out. We used a two channel portable recorder (Menfis Biomedical Inc., Bologna, Italy) connected to two glass pH-metric probes (Telemedicine srl., Naples Italy), which were introduced through the nose without any sedation, and placed 5 cm above and 10 cm below the upper and the lower edge of the LES, respectively. The percentage of total time of exposure to pH < 4 (normal value < 4.2%) was determined.

Twenty-four hours esophago-gastric bilimetry

Bilimetric evaluation was performed simultaneously with pH-metry. We utilized a portable recorder (BILITEC 2000, Sinectics Medical Inc.) connected to two optic-fiber probes placed 5 cm above and 10 cm below the upper and the lower edge of the LES, respectively. The percentage of total time of esophageal bilirubin absorbance > 0.14 (normal value < 7%) was determined.

Ethics committee approval

The ethics committee of the Second University of Naples approved our study and verbal consent was obtained from the study participants.

Statistical analysis

Values are expressed as mean ± SD. Data were compared using Student's *t*-test, Fischer's exact test, or the Chi-square test wherever appropriate. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Endoscopy

Endoscopic evaluation divided the 83 patients into 4 groups. Group Ⅰ consisted of 22 (26.5%) non-esophagitic patients, Group Ⅱ consisted of 24 patients (28.9%) with grade A-B esophagitis, Group Ⅲ consisted of 19 patients (22.8%) with grade C-D esophagitis, and Group Ⅳ consisted of 18 patients (21.6%) with Barrett's esophagus; of the latter, nine had short segment Barrett's esophagus (SSBE) and nine had long segment Barrett's esophagus (LSBE, Figure 1). Of the 83 patients, 61 (73.4%) had a hiatal hernia.

Symptoms

The analysis of the symptoms questionnaire showed that 71 patients (85.5%) had heartburn, 55 (66.2%) had regurgitation, and 44 (53%) had simultaneous heartburn and regurgitation. Twelve patients (14.4%) reported nocturnal cough and 7 (8.4%) reported chest pains. Analysis of symptom scores showed no significant between group differences. In contrast, symptom history was significantly higher in Group Ⅲ than in Groups Ⅰ and Ⅱ, but not between Barrett's patients (Figure 2).

Manometric data

Hypotonic LES was observed in 8 of 22 (36.3%) Group Ⅰ, 14 of 24 (58.3%) Group Ⅱ and 14/19 (73.7%) Group Ⅲ patients (Group Ⅰ *vs* Group Ⅲ: $P = 0.0279$). Hypotonic LES was also present in 8 of 9 (88.8%) LSBE and 7 of 9 (77.7%) SSBE patients. Mean LES-P in Group Ⅰ (13.91 ± 4.8 mmHg) was significantly higher than in Groups Ⅱ (9.2 ± 2.2 mmHg, $P < 0.001$), and Ⅲ (8.6 ± 2.8 mmHg, $P < 0.001$) and in SSBE (9.2 ± 3.4 mmHg, $P = 0.0056$) and LSBE (7.1 ± 1.6 mmHg, $P < 0.001$) patients.

Five of the 22 (22.7%) patients in Group Ⅰ showed ineffective esophageal motility, increasing to 50% (12/24) in Group Ⅱ and to 73.6% (14/19) Group Ⅲ.

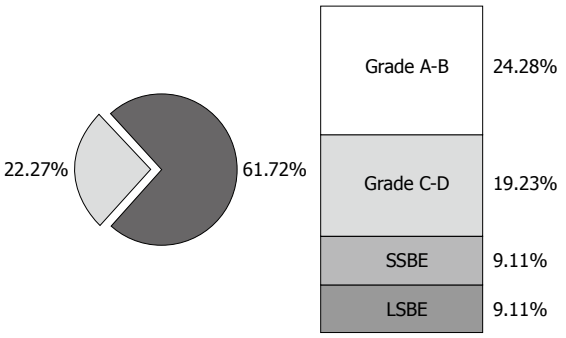

Figure 1 Endoscopy evaluation in 83 gastroesophageal reflux disease (GERD) patients. SSBE: Short segment Barrett's esophagus; LSBE: Long segment Barrett's esophagus.

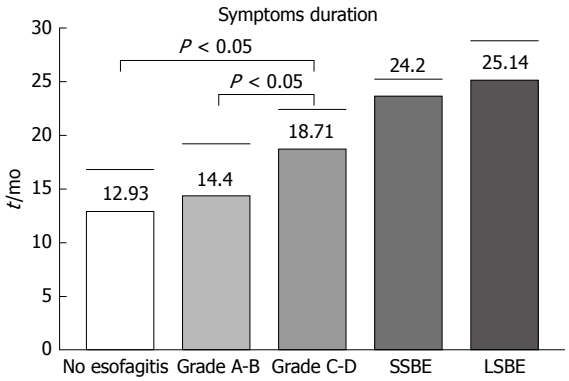

Figure 2 Symptoms. Mean duration of symptoms history in each group of GERD patients (mean ± SD, Fisher's exact test).

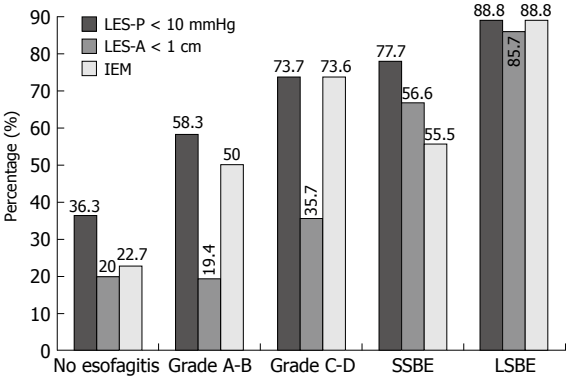

Figure 3 Manometry. Percentage with hypotonic lower esophageal sphincter (LES), Short LES and ineffective esophageal motility in each group of GERD patients (mean ± SD, Fisher's exact test).

In comparison, 5 of 9 SSBE (55.5%) and 8 of 9 (88.8%) LSBE patients showed ineffective esophageal motility (Figure 3).

pH-metric evaluation

The prevalence of pathologic acid reflux increased relative to esophagitis, from 45.4% (10/22) in Group I, to 66.6% (16/24) in Group Ⅱ and 73.6% (14/19) in Group Ⅲ. Six of 9 (66.6%) SSBE and 7 of 9 (77.7%) LSBE patients showed pathologic pH-metry (Figure 4).

Relative time at pH < 4 was 5.1 ± 2.7% in non-esophagitic (Group Ⅰ) patients, increasing to 7.03 ± 3.6% ($P > 0.05$) in Group Ⅱ and 7.2 ± 0.24% ($P > 0.05$) in Group Ⅲ. In contrast, both the SSBE (9.8 ± 5.1%, $P = 0.0022$) and LSBE (15.5 ± 7.7%, $P < 0.0001$) groups had significantly more time at pH < 4 than did Group Ⅰ (Figure 5).

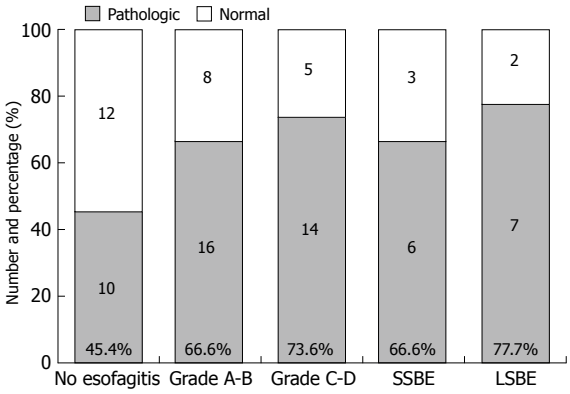

Figure 4 pH-metry. Number and percentage of pathologic and normal pH-metry in each group of GERD patients.

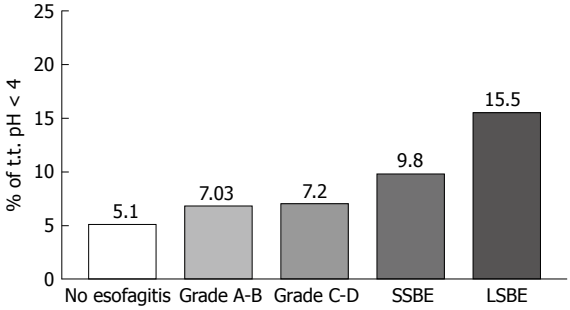

Figure 5 Esophageal acid exposure. Mean value of total time of esophageal exposure at pH < 4 in each group of GERD patients (mean ± SD).

Bilimetric evaluation

Pathologic bilirubin exposure was observed in 9 of 22 (53.3%) Group Ⅰ, 18 of 24 (75%) Group Ⅱ and 15 of 19 (78.9%) Group Ⅲ patients, as well as in 7 of 9 (77.7%) SSBE and 8 of 9 (88.8%) LSBE patients (Figure 6). The global prevalence of patients non-responsive to PPI therapy was 68.7% (57/83).

Mean time of bile absorbance > 0.14 in all patients was 16.9 ± 4.6%, 9.2 ± 5.2% in Group Ⅰ, 10.9 ± 4.6% in Group Ⅱ, 16.3 ± 6.3% in Group Ⅲ, 15.8 ± 6.7% in SSBE and 19.9 ± 6.2% in LSBE (Figure 7).

Combined pH-bilimetry evaluation

The analysis of combined pH-metry and bilimetry showed that 8 of 22 (36.4%) non-esophagitic patients and only 5 of 43 (11.6%) esophagitic patients [3 of 24 (12.5%) in Group Ⅱ and 2 of 19 (10.5%) in Group Ⅲ] had both values within the normal range. None of the 18 Barrett's patients had normal esophageal exposure to acid and bile.

Pathological bilimetry associated with normal pH-metry was observed in 4 of 22 (18.2%) non-esophagitic and 8 of 43 (18.6%) esophagitic patients [5 of 24 (20.8%) in Group Ⅱ and 3 of 19 (15.8%) in Group Ⅲ], as well

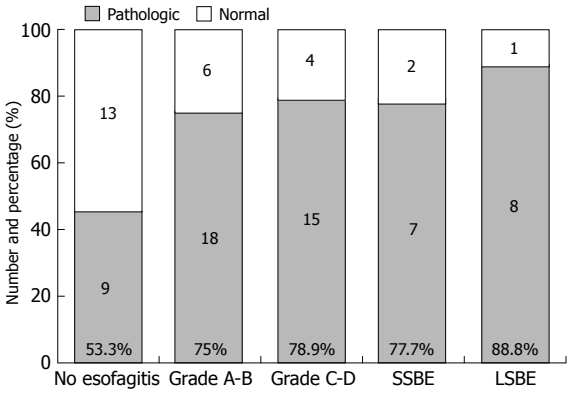

Figure 6 Bilimetry. Number and percentage of pathologic and normal bilimetry in each group of GERD patients.

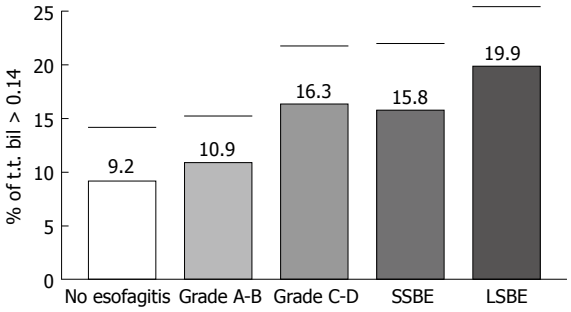

Figure 7 Esophageal bile exposure. Mean value of total time of esophageal bilirubin absorbance > 0.14 in each group of GERD patients (mean ± SD).

as in 3 of 9 (33.3%) SSBE and 2 of 9 (22.2%) LSBE patients.

Conversely, pathological pH-metry associated with normal bilimetry was observed in 5 of 22 (22.7%) non-esophagitic and 5 of 43 (11.6%) esophagitic patients (3 of 24 (12.5%) in Group Ⅱ and 2 of 19 (10/5%) in Group Ⅲ), as well as in 2 of 9 (22.2%) SSBE and 1 of 9 (11.1%) LSBE patients.

Pathologic bilimetry and pathologic pH-metry were observed in 5 of 22 (22.7%) non-esophagitic and 25 of 43 (58.1%) esophagitic patients [13 of 24 (54.2%) in Group Ⅱ and 12 of 19 (66.1%) in Group Ⅲ], as well as in 4 of 9 (44.4%) SSBE and 6 of 9 (66.7%) LSBE patients (Figure 8).

DISCUSSION

Although the introduction of PPIs has improved outcomes in GERD patients, a significant number of patients treated with a high dosage of PPIs (40 mg bid) show no improvements in symptoms or esophagitis. Of patients who do not respond to PPI therapy, however, only 37% show pathological pH-metry results[10]. In contrast, the combination of pH-metry and bilimetry showed pathological results in 70% of patients, thus improving the sensitivity of detection of reflux by 35%. These outcomes are important for the management of GERD patients, in that the constant presence of GERD symptoms, as documented by pH-metry, are probably caused by the incomplete acid-secretion control of the

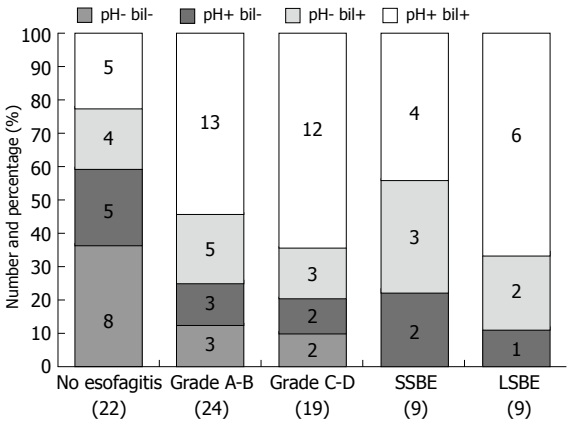

Figure 8 Combined pH-metry and bilimetry evaluation. Number and percentage of various associations of pH-metry and bilimetry in each group of patients.

PPI drugs. The presence of biliary reflux, as documented by bilimetry, suggests that the PPIs are unable to inhibit bile secretion. Persistent symptoms in patients without any documented evidence of acid and/or bile reflux suggests that these patients may be suffering from a less common disease, such as hypersensitive esophagus, or that these symptoms arise from psychiatric causes[12].

In this study, we analyzed patients non-responsive to an 8 wk course of high-dosage PPI therapy. In addition to assessing the presence of both acid and bile reflux, we assessed the features and duration of symptoms and the esophageal motor pattern. Our overall goal was to identify characteristics that could be linked to the persistent symptoms and typical lesions of GERD. We found that a high percentage of patients (36%) were poorly responsive to PPI therapy. When associated with a long symptom history, this characteristic showed a strong correlation with the presence of esophagitis. Compared with patients without esophagitis, those with esophagitis showed a significantly longer history of symptoms, but there was no differences in severity[13-14].

Functionally, manometric analysis has shown that hypotonic and short lower esophageal sphincter was correlated with esophagitis, with hypotonic and short LES having a strong influence on the natural history of GERD[15-16]. We found that these manometric alterations were present in only 36% of patients without esophagitis, increasing to 60% in patients with grade A-B esophagitis and to 70% in patients with grade C-D esophagitis. Moreover, in agreement with findings showing that effective esophageal motility (non peristaltic sequences and waves with amplitude < 15 mmHg) is important[16-17], we found that ineffective esophageal motility, while infrequent in non-esophagitic patients (22%), increased to 50% in patients with grade A-B esophagitis and to 73% in patients with grade C-D esophagitis.

Our findings also showed that a high percentage of GERD patients poorly responsive to PPIs have biliary reflux. We found that a high percentage (53.3%) off non-esophagitic GERD patients had pathologic bile reflux, increasing to 70% in esophagitic patients. In

addition, the percentage of total time of bile absorbance > 0.14 was associated with esophagitis severity.

It is important to emphasize that patients with severe GERD (i.e. presence of esophagitis and/or Barrett's esophagus) showed a significant increase in simultaneous bile and acid reflux relative to that in non-esophagitic GERD patients. Thus, in these patients, the esophageal mucosa is simultaneously exposed to the harmful effects of gastric and duodenal juice, with increased damage correlated with increased exposure. Similarly, animal models have shown that simultaneous exposure of the esophageal mucosa to both acid and bile reflux results in greater mucosal damage than exposure to isolated acid or bile reflux[17]. Moreover, while taurocholate does not cause mucosal damage at neutral pH, it does so at acid pH, as evidenced by ionic permeability studies[18].

On the contrary, the presence of a pathologic bile test without pathologic acid reflux, which was quite common in non-esophagitic patients and those with Grade A-B esophagitis, was observed in only 30% of patients with grade C-D esophagitis. This shows how the evolution of GERD to a more severe grade is influenced not only by acid reflux, but also by the association of acid reflux with duodenogastroesophageal reflux disease.

COMMENTS

Background

The available literature suggests that proton pump inhibitors (PPIs) are less efficacious in normalizing duodeno gastroesophageal reflux disease (DGERD), compared with their effect on acid reflux, in contrast to reflux surgery that has shown to adequately suppress both esophageal acid and bile exposure.

Research frontiers

This study clearly shows that the high percentage of patients poorly responsive to PPI therapy may result from poor control of DGERD. Many patients without esophagitis have simultaneous acid and bile reflux, which increases with increasing esophagitis grade.

Applications

Laparoscopic anti-reflux surgery seems to be the treatment of choice, being effective in suppressing both acid and bilirubin exposure.

Peer review

In this manuscript, the authors ascertained that many PPI-resistant GERD patients have simultaneous acid and bile reflux, which increases with increasing esophagitis grade. The study was well performed and the conclusion was clear.

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Does clamping during liver surgery predispose to thrombosis of the hepatic veins? Analysis of 210 cases

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Abstract

AIM: To test whether clamping during liver surgery predisposes to hepatic vein thrombosis.

METHODS: We performed a retrospective analysis of 210 patients who underwent liver resection with simultaneous inflow and outflow occlusion. Intraoperatively, flow in the hepatic veins was assessed by Doppler ultrasonography during the reperfusion phase. Postoperatively, patency of the hepatic veins was assessed by contrast-enhanced CT angiography, when necessary after 3-6 mo follow up.

RESULTS: Twelve patients (5.7%) developed intraoperative liver remnant swelling. However, intraoperative ultrasonography did not reveal evidence of hepatic vein thrombosis. In three of these patients a kinking of the common trunk of the middle and left hepatic veins hindering outflow was recognized and was managed successfully by suturing the liver remnant to the diaphragm. Twenty three patients (10.9%) who developed signs of mild outflow obstruction postoperatively, had no evidence of thrombi in the hepatic veins or flow disturbances on ultrasonography and contrast-enhanced CT angiography, while hospitalized. Long term assessment of the patency of the hepatic veins over a 3-6 mo follow-up period did not reveal thrombi formation or clinical manifestations of outflow obstruction.

CONCLUSION: Extrahepatic dissection and clamping of the hepatic veins does not predispose to clinically important thrombosis.

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Key words: CT-angiography; Doppler ultrasound; Liver resection; Pringle maneuver; Radiofrequency; Selective hepatic vascular exclusion

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INTRODUCTION

Vascular control during liver resection typically involves hepatic inflow occlusion, either continuous or intermittent. However, more complex resections may require both inflow and outflow occlusion, the latter usually being achieved with extraparenchymal control of the major hepatic veins at the hepatocaval junction. This maneuver can significantly reduce backflow bleeding during parenchymal transection and facilitate resection

of tumors close to the roots of the major hepatic veins and even reconstruction of a hepatic vein in the liver remnant[1].

Three techniques for vascular control have been widely used: the Pringle maneuver (PM), the selective hepatic vascular exclusion (SHVE) and the total hepatic vascular exclusion (THVE). The PM[2] is performed by encircling and clamping the hepatoduodenal ligament[1]. Although this is well tolerated, it should always be kept in mind that backflow bleeding and air embolization might occur during parenchymal transection[3]. THVE includes clamping of the hepatoduodenal ligament and occlusion of the suprahepatic and infrahepatic inferior vena cava (IVC)[4]. This technique has the advantage of a bloodless surgical field, but the serious hemodynamic instability it may cause, makes it inappropriate for 20%-30% of patients. SHVE entails disconnection of the liver from the retrohepatic IVC and inflow occlusion combined with extrahepatic control of hepatic veins. This technique offers bloodless liver transection without the above-mentioned disadvantages of PM and THVE[5,6].

Dissection and clamping of the hepatic veins during the application of SHVE may predispose the major hepatic veins to thrombi formation through the induction of venous stasis and endothelial injury coupled with coagulation disturbances. The scarcity of studies addressing this issue prompted us to test our hypothesis that dissection and clamping of the hepatic veins is associated with an increased risk of thrombosis and liver outflow obstruction.

MATERIALS AND METHODS

Between 1997 and 2007, 210 consecutive patients underwent hepatectomy with SHVE[1]. Briefly, in all cases and irrespective of the type of planned hepatectomy, the abdomen was accessed *via* a bilateral subcostal incision and the liver was fully mobilized after transection of its ligaments. The liver was then disconnected from the retrohepatic IVC by dividing the short perforator hepatic veins. On the right side, dissection along the anterior surface of the IVC continued until the right hepatic vein was isolated, while on the left side, the venous trunks of the left and middle hepatic veins were also dissected free from the surrounding tissues. Control of liver inflow was attained by clamping the porta hepatis with a Satinsky clamp and by occluding any accessory hepatic artery with bulldog clamps. Liver outflow control was achieved by clamping the trunks of the right, middle and left hepatic veins separately (Figure 1). The transection plane was defined with an intraoperative ultrasonographer (Aloka SSD-1400, model IP-1235V, ALOKA CO., LTD., Japan), in order to secure tumor-free margins > 1 cm. Liver splitting was performed using either the clamp crushing technique or by sharp transection with a knife. Hemostasis was achieved by suturing all vascular orifices on the cut surface with 3-0 and 4-0 prolene. After completion of the liver resection, outflow was released first, followed by liver inflow[5,6]. Following reperfusion, hemostasis was completed using additional stitches. Flow in the hepatic artery, the portal vein, the hepatic veins and the IVC was assessed by intraoperative Doppler ultrasonography. The negative findings in the first 120 consecutive patients prompted us to restrict our Doppler study to the hepatic veins. Thorough imaging of all liver vasculature was then reserved for cases with liver remnant swelling or signs of hyporeperfusion. All operations were performed by the same surgical team, directed by the senior author V.S. All patients had normal imaging of the hepatic veins before liver resection, either on contrast-enhanced CT or MRI.

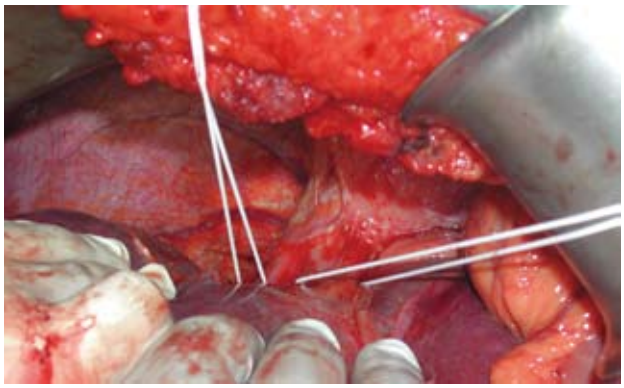

Figure 1 After careful dissection at the hepatocaval junction, the extrahepatic trunk of the right hepatic vein and the common trunk of the left and middle hepatic vein have been isolated and are ready to be clamped.

Postoperative Doppler ultrasonography of the portal vein, hepatic artery and the hepatic veins was performed at the bedside, if the patient exhibited at least one of the following findings: (a) clinically worsening ascites on any postoperative day; (b) persistent or worsening cholestasis (conjugated bilirubin > 3 mg/dL) after the 3rd postoperative day; (c) worsening elevation of transaminases after the 2nd postoperative day (AST and/or ALT > 1st postoperative day levels) or (d) persistent or worsening prolongation of INR > 2 after the 3rd postoperative day.

All patients admitted after 2002 (117 patients-56%) received perioperative thromboprophylaxis with low molecular weight heparin for a median duration of 10 d (range: 4-14 d), according to our new institutional protocol which was initiated at that time. Post-discharge, all patients were invited for a follow-up abdominal CT scan, 3-6 mo after surgery. Helical CT (Philips, The Netherlands) of the abdomen was performed before and after intravenous administration of iodinated contrast material. Contrast enhanced images were obtained in the arterial and portal venous phase and images were evaluated for the presence of hepatic venous thrombosis by two independent observers. Data collection was performed in a prospective manner.

RESULTS

Clinical, intraoperative and postoperative parameters of the patients are summarized in Table 1. Intraoperatively, 12 (5.7%) patients developed unexpected swelling of the liver remnant. Intraoperative Doppler ultrasonography

Table 1 Clinical, intraoperative and postoperative parameters of 210 patients undergoing liver resections under selective hepatic vascular exclusion (data are expressed as medians with range)

Clinical, intraoperative and postoperative parameters	
Age (yr)	63 (1-86)
Indication for hepatectomy	
Malignancy	173
Benign (hemangioma, hydatid cyst, *etc*)	37
Type of hepatectomy	
Right hepatectomy	92
Left hepatectomy	40
Minor hepatectomy (1-2 segments)	78
Intraoperative data	
Warm ischemic time (min)	39 (25-61)
Blood loss (mL)	420 (140-3100)
Transfusions of pRBCs (U)	0 (0-14)
Outcome	
Morbidity (pleural effusion, bile leak, chest infection, post-operative bleeding, wound infection, postoperative liver failure)	34%
Mortality	3 (1.5%)[1]
Hospital stay (d)	12 (4-37)

[1]One death due to postoperative liver failure, 1 due to cardiomyopathy and cardiac arrest and 1 due to postoperative sepsis.

did not reveal evidence of hepatic vein thrombosis in any of these cases. In three of these patients kinking of the common trunk of the middle and left hepatic veins was recognized and was managed by suturing the liver remnant to the diaphragm. In the remaining nine patients, extensive work-up did not show thrombi formation and the liver remnant swelling was attributed to the fact that the portal flow was disproportional to the small liver remnant.

Twenty three (10.9%) patients fulfilled the previously mentioned criteria for postoperative Doppler ultrasonography at the bedside. All these patients were also examined with contrast enhanced CT-angiography. The hepatic veins were visualized in all cases and no evidence of thrombosis was found.

Forty two (20%) patients underwent contrast-enhanced CT scans during their postoperative hospitalization for reasons unrelated to suspected hepatic vein thrombosis (diagnostic work-up for fever, bile collection and chest infection). In all cases, hepatic vein imaging did not reveal thrombi formation or recanalization processes.

Finally, 200 patients (95%) underwent a follow up contrast-enhanced CT scan of the liver at a median time of 150 d (range: 92-205 d) after surgery, without evidence of thrombotic processes or stricture of the hepatic veins.

DISCUSSION

Our study showed that dissection and clamping of the hepatic veins in hepatectomies under inflow and outflow vascular occlusion of the liver was not complicated with hepatic vein thrombosis either intraoperatively or postoperatively during a 6-mo follow-up period.

Postoperative hepatic venous outflow obstruction is extremely rare in large series of hepatectomies[7,8]. On the contrary, in liver transplantation the incidence is high, especially with the piggyback technique (0.5%-2.5%) which has a mortality rate of 24%[9-14]. The main causative factors are either technique-related or are associated with coagulation disturbances generated by the underlying disease and graft function.

The only study addressing the risk of hepatic vein thrombosis in liver resection is by Arita *et al*[15], who showed that 10 out of 821 liver resections performed using the intermittent Pringle maneuver developed hepatic vein thrombosis. It is worth noting that the authors had to resort to thrombectomy in the most severe cases. The exposure of major hepatic veins to a length of 3 cm or more and the use of an ultrasonic dissector were postulated to be predisposing factors for vein thrombosis.

Although our technique could be considered more thrombogenic, since it includes dissection and clamping of hepatic veins, the lack of thrombosis in our series is in surprising contrast to the findings of Arita *et al*, who performed only intermittent inflow occlusion. It is possible that the use of the ultrasonic dissection technique used by Arita *et al* could, as the authors themselves admitted, have contributed to the thrombogenic effect, which was further aggravated when the energy was delivered close to the major hepatic veins[15].

Our results are in agreement with the findings of most major clinical series of hepatectomies performed under vascular control, in which hepatic venous thrombosis is scarcely if ever mentioned[16,17]. Although SHVE could be considered more thrombogenic, since it involves injurious manipulation of hepatic veins, the lack of confirmed cases of vein thrombosis in our study can be attributed to short warm ischemic time and sharp transection of the liver surface with the scalpel, a technique that is less traumatic to venous epithelium compared to other ablative techniques. Avoidance of radiofrequency ablation in our series may also have contributed to our favorable results, since this technique has been recently associated with damage to the liver remnant[18] and hepatic vein thrombosis[19]. Venous endothelial trauma has been known to cause platelet aggregation and degranulation, vasoconstriction, thrombin activation and diminished fibrinolysis[20]. Therefore, we can not exclude the possibility that some of our patients may have developed small, undetected thrombi postoperatively. However, such thrombi remain clinically silent and resolve spontaneously without increasing morbidity or mortality. It is also possible that ischemia reperfusion of the liver mobilizes mechanisms that attenuate thrombi formation locally. Studies addressing the coagulation-fibrinolysis system during liver resection indicate that the balance leans towards fibrinolysis[21,22].

Regarding diagnosis of hepatic venous thrombosis, Doppler ultrasound is a readily available and inexpensive tool[23,24]. It is, however, operator-dependent and

its diagnostic accuracy may be compromised by the presence of bowel gas or ascites. On CT, vein thrombosis is seen as a lack of enhancement on post-contrast images, often associated with peripheral rim enhancement. In hepatic veno-occlusive disease, CT reveals patchy hepatic parenchymal enhancement with lack of normal visualization of the hepatic veins[25-27].

In conclusion, our analysis of a large cohort of patients confirms that extrahepatic dissection and clamping of the hepatic veins for up to one hour is a safe procedure that does not predispose to clinically important thrombosis. Although the technique of selective vascular exclusion used in our series is not advocated for routine use in liver surgery, we suggest that concerns about venous thrombosis are unjustified and should not be a limiting factor in the application of this useful technique, whenever necessary.

COMMENTS

Background

Dissection and clamping of the hepatic veins during liver resection may predispose the major hepatic veins to thrombi formation. In this study we test our hypothesis that dissection and clamping of the hepatic veins is not associated with an increased risk of thrombosis and liver outflow obstruction.

Research frontiers

Postoperative hepatic venous outflow obstruction is extremely rare in large series of hepatectomies. On the contrary, in liver transplantation the incidence is high, especially with the piggyback technique (0.5%-2.5%) which has a mortality rate of 24%. The main causative factors are either technique-related or are associated with coagulation disturbances generated by the underlying disease and graft function.

Innovations and breakthroughs

The only study addressing the risk of hepatic vein thrombosis in liver resection is by Arita *et al*, who showed that 10 out of 821 liver resections performed with the intermittent Pringle maneuver developed hepatic vein thrombosis. Our study showed that dissection and clamping of the hepatic veins in hepatectomies under inflow and outflow vascular occlusion of the liver was not complicated with hepatic vein thrombosis either intraoperatively or postoperatively during a six-month follow-up period.

Applications

Although the technique of selective vascular exclusion used in our series is not advocated for routine use in liver surgery, the authors suggest that concerns about venous thrombosis are unjustified and should not be a limiting factor in the application of this useful technique, whenever necessary.

Terminology

Pringle maneuver: performed by encircling and clamping the hepatoduodenal ligament; Selective hepatic vascular exclusion: inflow occlusion combined with extrahepatic control of hepatic veins; Total hepatic vascular exclusion: clamping of the hepatoduodenal ligament and occlusion of the suprahepatic and infrahepatic vena cava (IVC).

Peer review

This is an interesting paper about hepatic vein thrombosis.

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BRIEF ARTICLES

A study of pulmonary embolism after abdominal surgery in patients undergoing prophylaxis

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Abstract

AIM: To determine risk factors for pulmonary embolism and estimate effects and benefits of prophylaxis.

METHODS: We included 78 patients who died subsequently to a pulmonary embolism after major abdominal surgery from 1985 to 2003. A first, retrospective analysis involved 41 patients who underwent elective surgery between 1985 and 1990 without receiving any prophylaxis. In the prospectively evaluated subgroup, 37 patients undergoing major surgery between 1991 and 2003 were enrolled: all of them had received a prophylaxis consisting in low-molecular weight heparin, given subcutaneously at a dose of 2850 IU AXa/0.3 mL (body weight < 50 kg) or 5700 IU AXa/0.6 mL (body weight ⩾ 50 kg).

RESULTS: A higher incidence of thromboembolism (43.9% and 46.34% in the two groups, respectively) was found in older patients (> 60 years). The incidence of pulmonary embolism after major abdominal surgery in patients who had received the prophylaxis was significantly lower compared to the subjects with the same condition who had not received any prophylaxis ($P < 0.001$, OR = 2.825; 95% CI, 1.811-4.408). Furthermore, the incidence of pulmonary embolism after colorectal cancer surgery was significantly higher compared to incidence of pulmonary embolism after other abdominal surgical procedures. Finally, the incidence of pulmonary embolism after colorectal cancer surgery among the patients who had received the prophylaxis (11/4316, 0.26%) was significantly lower compared to subjects undergoing a surgical procedure for the same indication but without prophylaxis (10/1562, 0.64%) ($P < 0.05$, OR = 2.522; 95% CI, 1.069-5.949).

CONCLUSION: Prophylaxis with low molecular weight heparin is highly recommended during the preoperative period in patients with diagnosis of colorectal cancer due to high risk of pulmonary embolism after elective surgery.

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Key words: Pulmonary embolism; Surgery; Colorectal cancer; Risk factor; Prevention

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INTRODUCTION

Pulmonary embolism (PE) is a life-threatening condition or complication and might be one of the worst nightmares for most surgeons. PE is a partial obstruction of the pulmonary arterial three. The embolus that causes the obstruction usually travels through the venous system from a distant site. PE causes symptoms such as dyspnea, chest pain or collapse. Moreover, the clinical severity of PE can vary, ranging from asymptomatic cases to sudden death. Despite advances in diagnosis and treatment, PE remains a significant cause of morbidity and mortality and is still one of the most common preventable causes of

death, which is easily overlooked[1,2].

Risk factors for deep vein thrombosis (DVT) and PE are prior medical history of DVT or PE, recent surgery, general anesthesia lasting longer than 30 min, pregnancy, prolonged immobilization, age > 40 years, obesity or underlying malignancy[3,4]. Moreover, gynecologic surgery, major trauma and indwelling venous catheters are risk factors for DVT at any location. Otherwise, venous thrombosis commonly involves lower limbs, affecting most frequently calf veins, which are involved in virtually 100% of symptomatic, spontaneous lower extremity DVT. Although DVT usually starts in calf veins, it is propagated above the knee in 87% of symptomatic patients before the diagnosis has been made. However, more than 35% of patients who die from PE may have isolated calf vein thrombosis[5].

MATERIALS AND METHODS

Subjects

We identified 54690 patients who had surgery between January, 1985 and December, 2003. The study included 39 (50%) females and 39 (50%) males who died subsequently to a PE after major abdominal surgery throughout the study period.

Retrospective analysis

The database of the Institute for Digestive Disease, Clinical Center of Serbia was reviewed to identify patients who had undergone surgery from January, 1985 to December, 1990. Age, final pathologic diagnosis at authopsy, surgical procedures and venous thromboembolism prophylaxis modalities were recorded.

In a first retrospective evaluation, out of the 15427 patients who had undergone surgery between 1985 and 1990, we included 41 cases (20 men, 21 women) of PE confirmed at autopsy. These patients had not received any prophylaxis prior to elective surgery, as prophylaxis was not performed on a regular basis during that period in our country. The PE patients had undergone the following surgical procedures: total oesophagogastrectomy, 1 case (2,4%); total gastrectomy, 3 (7.3%); Billroth I -type gastric resection, 2 (4.8%); Billroth II-type gastric resection, 4 (9.7%); gastrostomy with jejunostomy, 1 (2.4%); fundoplication, 1 (2.4%); cholecystectomy, 5 (12.1%); ileal resection, 1 (2.4%); nephrectomy, 1 (2.4%); appendectomy, 2 (4.8%); inguinal hernioplasty, 2 (4.8%); adhesiolysis, 1 (2.4%); laparotomic exploration with biopsy of the tumor, 5 (14.6%); right hemicolectomy, 1 (2.4%); left hemicolectomy, 4 (9.7%); coecostomy, 1 (2.4%); splenectomy with distal pancreatectomy, 1 (2.4%); and low anterior resection of the rectum, 3 (7.32%).

Prospective analysis

In the second part of our study, a total of 39263 patients were admitted to the Institute of Digestive Disease, Clinical Center of Serbia, between January, 1991 and July, 2003, to undergo abdominal elective surgery. All patients who underwent major surgery received low-molecular weight heparin (LMWH) prophylaxis subcutaneously at a dose of 2850 IU AXa/0.3 mL (body weight < 50 kg) or 5700 IU AXa/0.6 mL (body weight ⩾ 50 kg), one hour before the surgery, 12 and 24 h after the main surgery, and once daily each hospital day after main surgery. Prophylaxis did not cause any side effects (e.g. bleeding). Out of this group, a total of 37 patients (19 men, 18 women) died after major surgery due to PE. Diagnosis was confirmed at autopsy. These patients had undergone the following major surgery procedures: total gastrectomy, 5 (13.5%); ulcer suture, 2 (5.4%); gastrostomy, 2 (5.4%); cholecystectomy, 4 (10.8%); hepatico-jejuno anastomosis according to Roux, 2 (5.4%); partial pericystectomy with omentoplasty, 2 (5.4%); ileal resection, 1 (2.7%); appendectomy, 1 (2.7%); hernioplasty, 1 (2.7%); abscess drainage, 1 (2.7%); laparotomic exploration with biopsy of the tumor, 2 (5.4%); total colectomy, 2 (5.4%); right hemicolectomy, 3 (8.1%); left hemicolectomy, 3 (8.1%), Dixon-type resection, 2 (5.4%); Hartman-type resection, 2 (5.4%); Belsey-type resection, 1 (2.7%) and double colostomy, 1 (2.7%) (Table 1).

Statistical analysis

Results were presented as mean ± SD or as stated. Distribution data were compared by χ^2 analysis or Kruskal-Wallis test, if data were not normally distributed. In addition, logistic regression tests were conducted. All statistical analyses were performed with the SPSS 10.0 for Windows package (SPSS Inc., Chicago, IL). Values at the $P = 0.05$ level were considered statistically significant.

RESULTS

No significant difference as to the gender distribution existed between the two groups (χ^2 test, $P > 0.05$). No significant difference in the mean age was found between patient groups (Kruskal-Wallis test, $P > 0.05$). However, a higher incidence of thromboembolism (43.9% and 46.34%) was found in older patients (60-69 year range) in both groups of patients.

Forty-one patients out of 15427 (0.27%) who did not receive prophylaxis developed a PE. Among the 39263 patients who received prophylaxis, 37 (0.09%) developed a PE in the postoperative period.

Among the 15427 cases evaluated retrospectively, we identified 4304 patients who underwent colorectal abdominal surgery. Of them, 1562 were cancer cases. Among colorectal cancer patients who underwent major abdominal surgery, 0.64% (10/1562) developed PE postoperatively, while the incidence of PE in all remaining patients was 0.11% (16/13865) ($P < 0.05$, OR = 5.577, 95% CI, 2.526-12.311).

Among the 39263 patients who had received prophylaxis before major surgery, 37 (0.09%) were diagnosed as having postoperative PE. Of the 39263 major cases evaluated prospectively, we identified 11735 patients who underwent colorectal abdominal surgery.

Table 1 Characteristics of patients with pulmonary embolism

Clinical data	PE without prophylaxis (n = 41)	PE with prophylaxis (n = 37)
Mean age, yr (range)	64 (26-79)	67 (45-79)
M/F	20/21	19/18
Cause of death (primary)		
Obstruction of right pulmonary artery	10	5
Obstruction of left pulmonary artery	3	5
Obstruction of pulmonary trunk	10	1
Obstruction of both right and left pulmonary arteries	11	20
Obstruction of pulmonary trunk plus both right and left pulmonary arteries	7	6
Cause of death (secondary)		
Colorectal malignancy	11	10
Other malignancy	14	12
Other diagnosis	16	15
Prophylaxis	None	LMWH *s.c*: 0.3 mL (BW ≤ 50 kg) or 0.6 mL (BW > 50 kg), 1 h before and 12 h after surgery
Time from surgery to death		
0-5 d	12	22
6-10 d	19	8
11-15 d	6	2
16-30 d	4	3
> 30 d	0	2

PE: Pulmonary embolism; LMWH: Low-molecular weight heparin.

Of them, 4316 were cancer cases. Among colorectal cancer patients who underwent major abdominal surgery, 0.25% (11/4316) developed PE postoperatively, while the incidence of PE in all remaining patients was 0.05% (17/34 947) ($P < 0.05$, OR = 5.250, 95% CI, 2.457-11.216).

The incidence of PE after colorectal cancer surgery among patients who had received prophylaxis was significantly lower compared to that observed in subjects with colorectal surgery due to carcinoma who had not received any prophylaxis, i.e. 0.26% (11/4316) *vs* 0.64% (10/1562) ($P < 0.05$, OR = 2.522; 95% CI, 1.069-5.949). Moreover, incidence of PE after major abdominal surgery of patients who had received prophylaxis was significantly lower compared to that seen in subjects with the same diagnoses who had not received any prophylaxis ($P < 0.001$, OR = 2.825; 95% CI, 1.811-4.408).

DISCUSSION

PE is third most common cause of death in the US, with at least 650 000 cases occurring annually. Furthermore, PE represents the first or second most common cause of unexpected death in most age groups. The highest incidence of recognized PE occurs in hospitalized patients. Autopsy results are showing that up to 60% of patients who die at a hospital have PE, and that diagnosis is missed in about 70% of cases[6]. The annual incidence of known DVT and PE in the Western world is 1.0 and 0.5 per 1000, respectively. There are 65 000 cases each year among hospital patients in England and Wales. The prevalence of unsuspected PE diagnosed at autopsy is 3%-8%, and has been unchanged for 3 decades.

PE is common during all trimesters of pregnancy and puerperium, and incidence of PE is increasing with oral contraceptive or hormone replacement therapy. However, sex alone is not an independent risk factor[7].

Although the frequency of PE increases with age, this is not independent risk factor. Nevertheless, the accumulation of different risk factors, such as underlying illnesses and decreased mobility, increases the frequency of PE in older patients. Unfortunately, diagnosis of PE is often missed, especially in older patients. PE is diagnosed in 30% of all patients who die with massive PE, but only in 10% of those who are 70 years of age or older. Thus, PE still remains the most commonly missed diagnosis in the elderly institutionalized patients[7].

In our study, we found a higher incidence of PE in older patients (> 60 years of age) in both groups (43.9% and 46.34%).

Surgical patients have long been recognized to be at special risk for DVT and PE, but these problems are not confined to surgical patients. Surgeons should always suspect PE in case of a sudden circulatory collapse occurring within one to two weeks after surgery.

The risk of postoperative venous thromboembolism is reported to be twice as high in patients with cancer compared of those without cancer undergoing comparable surgery[8]. This risk is also higher in patients undergoing surgery for colorectal cancer as compared to those having abdominal surgery without malignancy. Thromboembolic complications are responsible for about half of deaths following elective colorectal surgery[9]. The highest rate (1.8%) of fatal PE was reported in patients following colorectal surgery, with a 3.3-fold increase compared to the overall rate observed among surgical patients, according to a retrospective 10-year review from Switzerland[10]. In this study, the increased risk of PE can be explained by a number of factors, such as malignancy-related hypercoagulable

state, postoperative infectious complications, prolonged surgery, pelvic dissection *etc*[11].

Overall, the incidence of PE after general surgery observed in Japan was 0.33%. Fatal PE was reported in 0.08% of the surgical population and the mortality rate of patients with PE was 31%. In addition, the incidence of PE after cancer surgery ranged from 0.57% after colon malignancy to 3.85% after pancreatic cancer surgery, and was significantly higher than in non-cancerous conditions (0.20%)[12].

An increased risk of PE after colorectal surgery has also been showed by Lee *et al* in a study on Chinese patients who underwent colorectal surgery without DVT prophylaxis. The authors demonstrated the occurrence of asymptomatic calf vein thrombosis in 41.7% of patients using serial Duplex ultrasound studies[13].

In our experience, the incidence of PE after colorectal cancer surgery was significantly higher compared with other surgical procedures. However, the incidence of PE after colorectal cancer surgery of patients who received prophylaxis was significantly lower compared to that seen among subjects with colorectal surgery due to carcinoma without prophylaxis.

In the study by Shukla *et al*[11], 99 patients with colorectal cancer selected for surgery were included. Fifty-one patients were randomized to receive LMWH while 48 patients did not receive any prophylaxis. At the end of the study, neither DVT nor PE cases were observed[12].

Anticoagulant prophylaxis is effective in preventing PE in hospitalized patients, since it reduces mortality after surgery. Prophylaxis with LMWH leads to effective reductions in the incidence of DVT after abdominal surgery in patients at risk for thromboembolic complications.

Initial treatment with LMWH following oral anticoagulant therapy with INR ranging from 2 to 3 was associated with an incidence of major bleeding of 3% at 3 mo while the mortality rate was 0.3%[14].

However, Diener *et al*, showed that there may be a dose-dependent risk of bleeding with LMWH therapy[15]. Low dose of LMWH was arbitrarily defined as a fixed dose of less than 6000 IU daily, whereas any higher dose of LMWH was considered as LMWH high dose. Concerning weight-adjusted doses of LMWH, 86 IU/kg per day was considered as LMWH low dose, while 86 IU/kg twice a day was considered as LMWH high dose.

In our study, patients who received prophylaxis with low dose LMWH after major abdominal surgery did not have any side effects (such as bleeding). Moreover, incidence of PE was significantly lower compared to subjects with the same conditions who did not receive prophylaxis.

The incidence of PE was four to six times lower in patients who had mechanical prophylaxis, although the difference was not significant. Preoperative prophylaxis for DVT is important, but further research is needed to estimate its effects and benefits[12].

In our study, older age (> 60 years) was identified to be a risk factor for PE. Prophylaxis with LMWH is highly recommended for patients with colorectal cancer before major surgery. As the mortality from PE depends on correct and timely diagnosis, it is of the utmost importance for clinicians to consider this possibility and perform proper diagnostic tests, especially in patients with colorectal cancer.

COMMENTS

Background

Pulmonary embolism (PE) is a life-threatening condition or complication and might be also one of the worst nightmares for most surgeons. Despite advances in diagnosis and treatment, PE remains a significant cause of morbidity and mortality and is still one of the most common preventable causes of death, which is easily overlooked. Risk factors for deep vein thrombosis (DVT) and PE are prior medical history of DVT or PE, recent surgery, general anesthesia lasting longer than 30 min, pregnancy, prolonged immobilization, age > 40 years, obesity or underlying malignancy.

Research frontiers

PE is the third most common cause of death in the US, with at least 650 000 cases occurring annually. Furthermore, PE represents the first or second most common cause of unexpected death in most age groups. The highest incidence of recognized PE occurs in hospitalized patients. The highlight of this article was to characterize relationship between PE and prophylaxis with a low dose of low-molecular weight heparin.

Innovations and breakthroughs

The highest rate of fatal PE in previous studies was reported in patients following colorectal surgery. Shukla *et al* described that increased risk of PE has been attributed to a number of factors such as malignancy-related hypercoagulable state of cancer patients, postoperative complications due to infections, prolonged surgery and pelvic dissection. In our experience, incidence of PE after colorectal cancer surgery was also significantly higher compared with other surgical procedures. However, in our study, patients who received prophylaxis with low dose low-molecular weight heparin (LMWH) after major abdominal surgery did not have any side effects (such as bleeding). Moreover, the incidence of PE was significantly lower compared to subjects who did not receive the prophylaxis.

Applications

The results of this study suggest that prophylaxis with LMWH is highly recommended for older patients (> 60 years) and patients with colorectal cancer before major surgery. As the mortality from PE depends on a correct and timely diagnosis, it is of the utmost importance for clinicians to consider this possibility and perform proper diagnostic tests, especially in patients with colorectal cancer.

Terminology

Prophylaxis: A low dose of LMWH was arbitrarily defined as a fixed dose of less than 6000 IU daily. A dose of LMWH above 6000 IU was considered high dose LMWH. Concerning weight-adjusted doses LMWH, 86 IU/kg per day was considered as LMWH low dose while 86 IU/kg twice a day was considered LMWH high dose.

Peer review

This controlled study shows that prophylaxis with low dose of LMWH significantly decreases the incidence of PE after surgery. In addition, our research may foster new therapeutic developments in the treatment of PE.

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Usefulness of magnifying endoscopy in post-endoscopic resection scar for early gastric neoplasm: A prospective short-term follow-up endoscopy study

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Abstract

AIM: To investigate the relationship between post-endoscopic resection (ER) scars on magnifying endoscopy (ME) and the pathological diagnosis in order to validate the clinical significance of ME.

METHODS: From January, 2007 to June, 2008, 124 patients with 129 post-ER scar lesions were enrolled. Mucosal pit patterns on ME were compared with conventional endoscopy (CE) findings and histological results obtained from targeted biopsies.

RESULTS: CE findings showed nodular scars (53/129), erythematous scars (85/129), and ulcerative scars (4/129). The post-ER scars were classified into four pit patterns of sulci and ridges on ME: (Ⅰ) 47 round; (Ⅱ) 54 short rod or tubular; (Ⅲ) 19 branched or gyrus-like; and (Ⅳ) 9 destroyed pits. Sensitivity and specificity were 88.9% and 62.5%, respectively, by the presence of nodularity on CE. Erythematous lesions were high sensitivity (100%), but specificity was as low as 36.7%. The range of the positive predictive value (PPV) on CE was as low as 10.6%-25%. Nine type Ⅳ pit patterns were diagnosed as tumor lesions, and 120 cases of type Ⅰ-Ⅲ pit patterns revealed non-neoplastic lesions. Thus, the sensitivity, specificity, and the PPV of ME were 100%.

CONCLUSION: ME findings can detect the presence of tumor in post-ER scar lesions, and make evident the biopsy target site in short-term follow-up. Further large-scale and long-term studies are needed to determine whether ME can replace endoscopic biopsy.

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Key words: Endoscopic mucosal resection; Endoscopic submucosal dissection; Magnifying endoscopy; Pit pattern; Scar

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INTRODUCTION

The use of magnifying endoscopy (ME) is now being reassessed since the successful study led by Professor Kudo regarding the utilization of magnifying colonoscopy[1]. Indeed, ME procedures for the upper gastrointestinal tract have been developed that make it possible to perform a variety of assessments, from routine

observation to a detailed examination of squamous dysplasia, squamous-cell carcinoma, Barrett's esophagus and associated dysplasia/early cancer, gastric cancer, and *Helicobacter pylori* infection[2-4]. ME with a narrow band image can aid in deciding the target of endoscopic biopsy for surveillance in Barrett's esophagus[5-8]. The relationships between ME findings and gastric neoplastic histology, including the types of cancer detected, are now being investigated, and the usefulness of ME for diagnosing early gastric cancer has been reported[9-15].

Little data, however, are currently available regarding the correlation between the findings of ME and pathological findings in post-endoscopic resection (ER) scars. There has been no definitive endoscopic description of which endoscopic findings need endoscopic biopsy or where the endoscopist has to target the biopsy in altered large scar lesions. In addition, it remains controversial as to whether a biopsy should be performed for each endoscopy in patients who have already undergone complete ER. Thus, we have evaluated the relationship between the real-time diagnosis of post-ER scars observed by ME and the pathological diagnosis, thereby validating the clinical usefulness of ME as a follow-up method for post-ER scars in early gastric neoplasm.

MATERIALS AND METHODS

Patients and definition

From January, 2007 to June, 2008, a total of 143 lesions (138 patients) underwent endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) in our hospital (Cheonan Hospital, Soonchunhyang University). "*En bloc* resection" is defined as the resection of a single piece as opposed to piecemeal resection in multiple pieces. "Complete *en bloc* resection" is defined as a lesion being contained within the mucosal layer, with no lympho-vascular invasion, and all margins (deep and lateral) histologically demonstrated to be tumor-free.

Among 138 patients, 8 patients who had been revealed as incomplete ER were excluded because they received a subsequent operation. Other exclusion criteria were as follows: (1) refusal to participate in the study (3 cases); (2) recurrent tumorous lesions (1 case); (3) NSAIDs or anticoagulant drug users (2 cases). A total of 129 lesions (124 patients) were finally enrolled in this study. All patients provided written informed consent, and the clinical study was performed according to guidelines approved by the ethics committee of Soonchunhyang Cheonan Hospital. No patients were lost during follow-up.

Methods

For the endoscopic examination, we used a GIF-Q240Z video endoscope (Olympus Optical Co., Ltd, Tokyo, Japan) fitted with an optic-type zoom lens that provided up to 80 times magnification and a high-resolution color charge-coupled device (CCD) connected to a 14-inch monitor. A transparent tip attachment (D-201-11802; Olympus) projecting 2 mm from the endoscopic tip was pressed against the mucosa in order to maintain good focus. To decrease the influence of mucus in the stomach under magnified observation, all patients ingested simethicone (20-30 mL), and a mucolytic agent (10% N-acetylcysteine 20 to 30 mL) was sprayed on the mucosal surface[4]. Evaluation of the entire stomach was initially performed with conventional endoscopy (CE) to exclude obvious lesions and to define scar lesions. Next, with the endoscope positioned on the scar lesions, complete magnification was obtained, with particular attention being paid to minute surface architecture and arrangements. Following complete identification, targeted biopsy specimens of the scar lesions were obtained. Conventional and magnifying endoscopic procedures were performed by an endoscopist with 10 years endoscopic experience. All examinations and images were digitally stored and documented on commercially available videotapes. Classification and analysis of the magnified view were carried out using the photographs and recorded videos by another endoscopist who was blinded to the examinations and histopathologic results. When pit patterns were mixed, classification was based on the most prominent pattern. We performed an endoscopic biopsy on sites with prominent or higher grade pit patterns. Following ER, all patients were given a PPI (omeprazole 40 mg) for eight weeks. Conventional and magnifying endoscopies were performed with the targeted biopsy of all scar lesions two months after the ER.

Classification by conventional and magnifying endoscopy

We classified CE characteristics of scar lesions according to the following attributes: height (elevated, flat, or depressed); nodularity (non-nodular or nodular); color (erythematous, pale, or iso-color with the surrounding mucosa); and ulceration (present or absent) (Figure 1). Next, the mucosal pit patterns in the post-ER scars were observed closely using ME. The mucosal pits were classified into four patterns of sulci and ridges: (Ⅰ) round pit patterns; (Ⅱ) short rod or tubular pit patterns; (Ⅲ) branched or gyrus-like pit patterns; and (Ⅳ) destroyed pit patterns (Figure 2). The criteria for suspecting a tumorous lesion included the observation of a fundamentally destroyed pit pattern (Type Ⅳ).

Histological assessment

The curative potential of *en bloc* resection was carefully evaluated histopathologically; slices were made at 2 mm intervals according to the Japanese Classification of Gastric Carcinoma[16]. Following magnifying observation, standard histological assessment was performed with H&E staining. Lesions were classified into four groups for diagnostic purposes: non-neoplastic lesions, low-grade adenomas, high-grade adenomas, and carcinomas. These diagnostic criteria were based on the Vienna classification of gastrointestinal epithelial neoplasia[17]. The histological type and the degree of various pathologic findings were evaluated to determine the relationship between endoscopic findings such as foveolar hyperplasia,

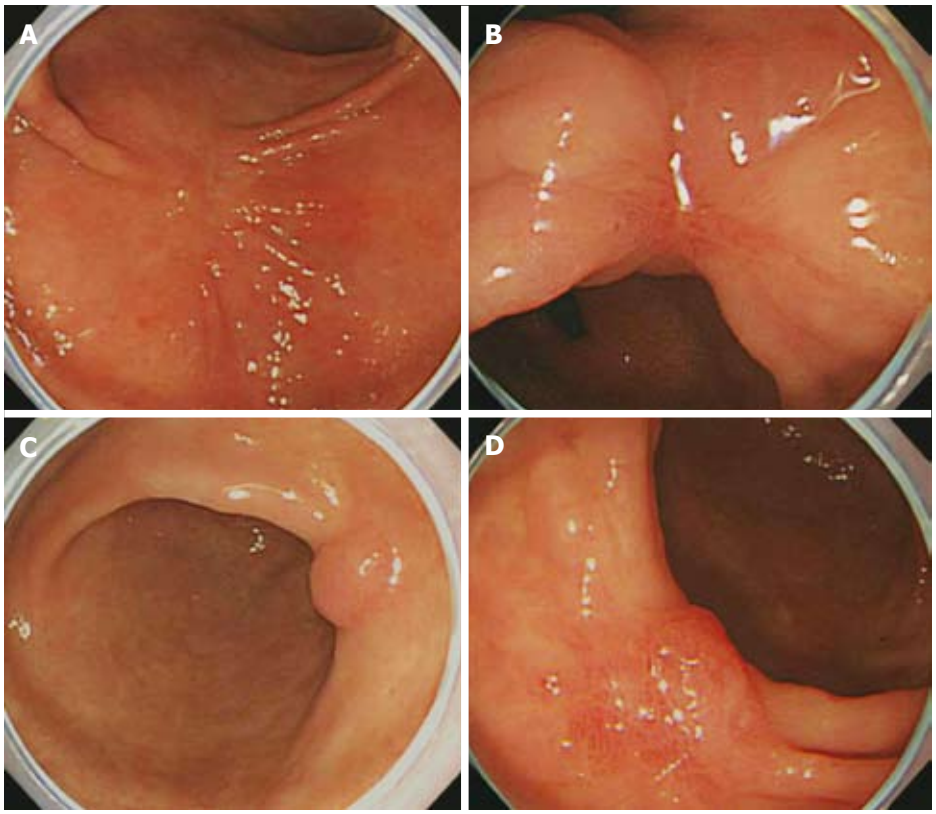

Figure 1 Conventional endoscopic characteristics of scar lesions. A: Height; flat, non-nodular and iso-color; B: Height; depressed, non-nodular and erythematous color; C: Height; elevated, non-nodular, and iso-color; D: Height; elevated, distorted nodular, and erythematous color.

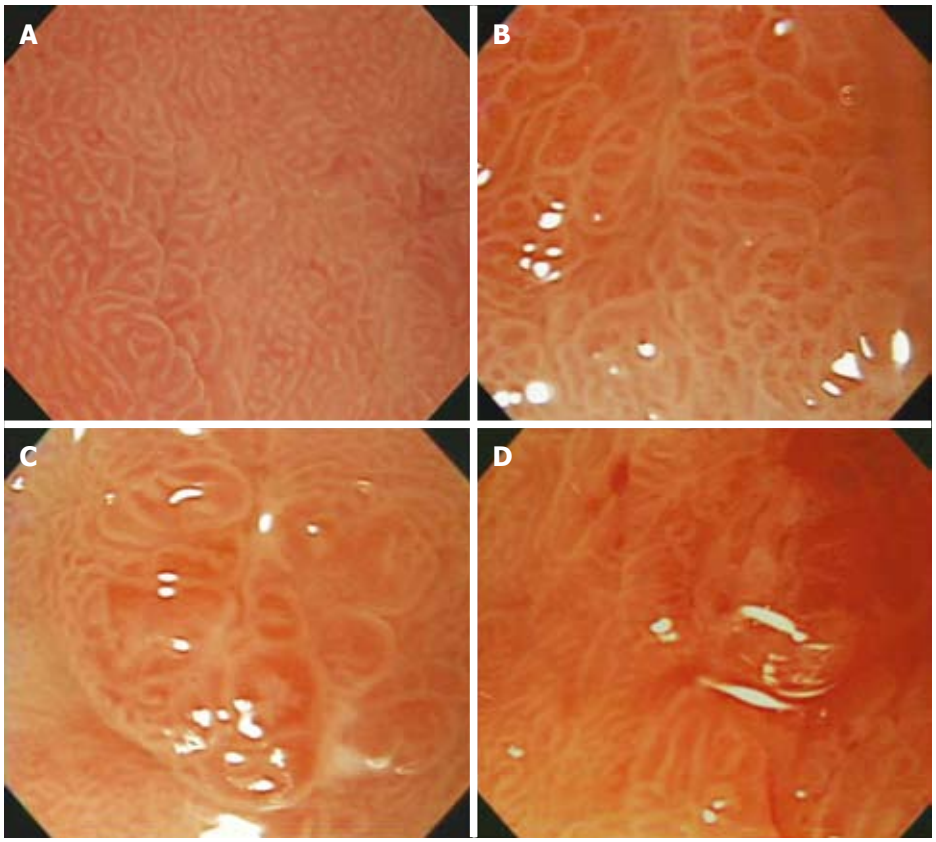

Figure 2 Magnifying images of scar lesion showing fine mucosal pit patterns. The four pit patterns of sulci and ridges identified are as follows: A: Type Ⅰ pit; small round, normal-like pit pattern; B: Type Ⅱ pit; short rod or tubular pit pattern; C: Type Ⅲ pit; branched or gyrus-like pit pattern; D: Type Ⅳ pit; destroyed pit pattern.

congestion of glands, atrophy, intestinal metaplasia, and fibrosis. These findings were then classified and scored from 0 to 3, respectively (0 = normal, 1 = mild, 2 = moderate, 3 = severe). A single pathologist who was blinded to the endoscopic findings reviewed and scored all the biopsy specimens. All pathologic findings were then compared in terms of both CE and ME findings.

Statistical analysis

Statistical evaluations were performed using SPSS

Table 1 Patient characteristics

Parameter	
Case (Patient)	129 (124)
Male	79
Female	45
Age, yr (SD)	58.51 (11.27)
Male	59.48 (11.79)
Female	56.71 (10.13)
ER outcome	
ESD/EMR	104/25
Complete resection	123
En bloc	117
Piecemeal	6
Incomplete resection	6
Post-ER diagnosis	
Adenoma	43 lesions
Adenoma with HGD	48 lesions
Adenocarcinoma	38 lesions
Follow up mo (SD)	2.27 (0.46)

SD: Standard deviation; ER: Endoscopic resection; ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection; HGD: High grade dysplasia.

statistical software, version 12.0 (SPSS Inc., Chicago, IL, USA). The Chi-square test was used to compare categorical variables and the ANOVA test was used to compare continuous variables. A P value of less than 0.05 was considered statistically significant.

RESULTS

Outcomes and histological diagnosis of the ER

Among 129 lesions, the method of ER was 104 ESD and 25 EMR. Complete resection was performed in 123 cases [117 *en bloc* resections (93 ESD and 24 EMR) and 6 piecemeal resections (2 ESD and 4 EMR)]. Six patients with incomplete resection who declined surgery were included in a follow-up endoscopic study. A set of 129 lesions from 124 patients were confirmed histologically by ER as consisting of 38 adenocarcinomas, 48 high-grade adenomas, and 43 low-grade adenomas. Following ER, the mean follow-up time was 2.27 ± 0.46 mo (mean ± SD) (Table 1).

Conventional and magnifying findings in post-ER scars

The CE findings revealed the following results: 38 elevated, 79 flat, and 12 depressive-type scars; 76 non-nodular and 53 nodular scars; 85 erythematous and 44 pale or iso-colored scars; and 4 ulcerative scars. The minute surface structure of post-ER scars, as shown by ME, demonstrated four pit patterns of sulci and ridges. These pit patterns were classified according to the main pit pattern as follows: (Ⅰ) 47 round pit patterns; (Ⅱ) 54 short rod or tubular pit patterns; (Ⅲ) 19 branched or gyrus-like pit patterns; and (Ⅳ) 9 destroyed pit patterns. There was no statistical significance between conventional endoscopic and ME findings ($P > 0.05$), although the presence of nodularity and erythematous lesions was high in type Ⅲ or IV pit patterns on ME ($P = 0.091$, $P = 0.079$, respectively, Table 2).

Table 2 Endoscopic findings and pit pattern in post-ER scar

CE findings	Pit type (No.)				Total (129)	*P* value
	Ⅰ (47)	Ⅱ (54)	Ⅲ (19)	Ⅳ (9)		
Height						0.205
Elevated	14	20	3	6	38	
Flat	29	29	13	3	79	
Depressed	4	5	3	0	12	
Nodularity						0.091
Present	17	18	11	6	53	
Absent	30	36	8	3	76	
Color						0.079
Erythematous	30	31	16	8	85	
Iso or pale	17	23	3	1	44	
Ulceration						0.285
Present	0	2	1	1	4	
Absent	47	52	18	8	125	

CE: Conventional endoscopy; Ⅰ: Round pit; Ⅱ: Short rod or tubular pit; Ⅲ: Branched or gyrus-like pit; Ⅳ: Destroyed pit pattern.

Endoscopic findings and pathologic features

Eight lesions revealed the presence of tumors in 53 cases with nodularity, while one lesion had no nodularity in the post-ER scar. Nine lesions revealed the presence of tumors in 85 cases with erythematous lesions. One lesion revealed the presence of tumors in 4 cases with non-healed ulcer lesions. Sensitivity and specificity were 88.9% and 62.5%, respectively, when the presence of nodularity aided in the detection of a neoplastic lesion on CE. Erythematous lesions had a high sensitivity (100%), but specificity was as low as 36.7%. The presence of an ulcer had low sensitivity (11.1%) and high specificity (97.5%). The range of the positive predictive value was as low as 10.6%-25% (Table 3). As assessed by CE, none of the mucosal height terms, color, nodularity, or ulceration showed statistical significances between various non-neoplastic pathologic features in post-ER scars.

Although there was no statistical significance in the relationship between endoscopic findings and other non-neoplastic pathologic findings, type Ⅲ or Ⅳ pit patterns exhibited slightly higher histological scores in terms of gland congestion, intestinal metaplasia, and atrophy ($P > 0.05$, Table 4). Nine type Ⅳ pit patterns on ME were diagnosed as tumor lesions, pathologically, and 120 cases of type Ⅰ-Ⅲ pit patterns revealed non-neoplastic lesions without tumor lesions. Thus, the sensitivity, specificity, and the positive predictive value were 100%, 100% and 100%, respectively (Table 5). Six cases were noted in patients who had received incomplete resection. Three cases of piecemeal resection for early gastric cancer were diagnosed as tumor lesions in spite of histologically complete resection.

DISCUSSION

Magnifying colonoscopy has already been reported as a clinically useful tool for diagnosing colorectal tumors[1,18]. Furthermore, ME has been confirmed as being superior to conventional colonoscopy with respect to its predictive

Table 3 Endoscopic findings and pathologic result in post-ER scar

CE findings (No.)	Pathologic results					
	Non-neoplastic	Neoplastic	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
Presence of nodularity (53)	45	8	88.9 (68.4-100)	62.5 (53.8-71.2)	15.1 (5.5-24.7)	98.7 (96.1-100)
Erythematous lesion (85)	76	9	100.0	36.7 (28.0-45.3)	10.6 (4.0-17.1)	100.0
Presence of ulcer (4)	3	1	11.1 (0.0-31.6)	97.5 (94.7-100)	25.0 (0.0-67.4)	93.6 (89.3-97.9)
Total No.	120	9				

PPV: Positive predictive value; NPV: Negative predictive value; CE: Conventional endoscopy.

Table 4 Pit pattern and non-neoplastic pathologic findings (mean score) in post-ER scar

Pit type	Foveolar hyperplasia	Gland congestion	Intestinal metaplasia	Atrophy	Fibrosis
Ⅰ	0.96	0.64	1.09	1.11	0.21
Ⅱ	0.96	0.48	1.11	1.11	0.19
Ⅲ	0.84	0.79	1.05	1.26	0.11
Ⅳ (coexisting findings with tumor)	0.78	0.89	1.33	1.56	0.33
P value[1]	0.837	0.244	0.828	0.344	0.644

Scored from 0 to 3, respectively (0 = normal, 1 = mild, 2 = moderate, 3 = severe). [1]Statistical significances were tested by one-way analysis of variance.

Table 5 Pit pattern and pathologic result in post-ER scar

Pit type	Pathologic results	
	Non-neoplastic	Neoplastic
Ⅰ: Round	47	0
Ⅱ: Short rod or tubular	54	0
Ⅲ: Branched or gyrus-like	19	0
Ⅳ: Destroyed[1]	0	9
Total No.	120	9

[1]Sensitivity: 100%; Specificity: 100%; Positive predictive value: 100%.

power for diagnosing pathological neoplasms detected during endoscopy. Technological improvements in recent years have demonstrated that ME can identify the fine mucosal patterns of the gastrointestinal tract, and it is now evident that the findings obtained from this new procedure correlate positively with histological findings[19,20], and that ME can help determine the target biopsy site during surveillance in Barrett's esophagus[5-8]. Despite these advances, however, there have been few studies investigating whether ME is capable of improving the rate of prediction for pathological diagnosis of gastric scar lesions after ER in early gastric neoplasm beyond that of the conventional method. To the best of our knowledge, this is the first description in the English literature of magnifying endoscopic classification and the characteristic definition of gastric post-ER scar lesions which includes comparative pathology for both magnifying and conventional procedures.

Diagnosis of early gastric cancer relies on macroscopic findings by CE, namely flat, elevated, or depressed; color identical to the neighboring noncancerous area, red or pale; the presence of granules or nodules; the presence or absence of ulcers; and the presence or absence of fold conversions, among others[13]. During diagnostic endoscopy, the endoscopist usually takes routine biopsies from even inconspicuous lesions that appear slightly erythematous, discolored, flat, granular, or shallow depressed mucosal areas in the stomach[14,21]. There has been no definitive description of which findings require endoscopic biopsy or where we have to target the biopsy in post-ER scar lesions in early gastric neoplasm. In our study, nodularity and erythematous lesions revealed the presence of tumors (sensitivity, 88.9% and 100%, respectively). One of 4 ulcer lesions revealed the presence of tumor (sensitivity, 11.1%). These CE findings were important in differentiating tumors in post-ER scars, but these findings in post-ER scar lesions are not specific to tumorous lesions (positive predictive value: 10.6-25.0%) in terms of diagnosing recurrence or suspected tumor in this study. Additionally, these findings give no specific information as to where we must target biopsies in certain large post-ER scar lesions. We cannot ignore endoscopic biopsy in cases with these endoscopic findings, which requires additional costs and is invasive in certain cases with no tumorous post-ER scar lesions.

Nevertheless, there is controversy regarding whether endoscopists should perform a biopsy during every follow-up study after complete ER. Recently, several endoscopists have suggested that short-term endoscopic examination is not necessary since complete ESD was introduced[22]. With recent advances in endoscopic skill and equipment, gastric neoplasms can be resected more completely by ESD, a technique that can produce larger and safer margins around the tumor compared to conventional EMR, thus making the rate of tumor recurrence very low. Recent ESD results have achieved greater than 95% *en bloc* resection as well as excellent survival rates[23,24]. In short-term follow-up endoscopic examinations in post-ER scars, the presence of a tumor can be considered residual tumor rather than the recurrence of a new tumor when we consider the doubling time of early gastric neoplasm.

Using ME, we classified post-ER scar lesions according to the fine gastric mucosal pit patterns of sulci and ridges as follows: (Ⅰ) round pit patterns; (Ⅱ) short rod or tubular pit patterns; (Ⅲ) branched or gyrus-like pit patterns; and (Ⅳ) destroyed pit patterns. Non-tumorous lesions in post-ER scars included type Ⅰ, Ⅱ, and Ⅲ pit patterns, and none of these pit patterns were identified as histologically discernable tumorous lesions

in our study. All the tumor lesions were noted in post-ER scar lesions with the type Ⅳ pattern. Our results suggest that the ME pattern may be considered a useful diagnostic tool capable of replacing the more invasive technique of endoscopic biopsy or identifying the target biopsy site in cases with mixed pit patterns.

Although there was no statistical significance in the relationship between endoscopic findings and other non-neoplastic pathologic findings, type Ⅲ or Ⅳ pit patterns exhibited slightly higher histological scores in terms of gland congestion, intestinal metaplasia, and atrophy. High scores with regard to gland congestion may play a role in the regeneration process, and high scores with regard to the other two findings may be suspected in relation to pathology near the original gastric neoplasm before ER. We were unable to evaluate whether these findings demonstrated a tendency toward tumor development. A longitudinal long-term follow-up study is needed to determine the significance of these non-neoplastic pathologic findings, and a large-scale study is needed to assess the relationship between these pathologic findings and the presence of tumors in post-ER scar lesions.

In this study, none of the patients who had been treated with complete *en bloc* resection by ESD had the type Ⅳ pattern, and no tumor lesions were observed pathologically in these patients. Nine tumor lesions were noted in cases with incomplete resection (6 cases) and piecemeal resection by EMR (3 cases). Consequently, we believe that ME will be useful in predicting the pathological diagnosis of tumorous lesions in post-ER scars, especially after incomplete or piecemeal resection. Furthermore, compared with CE, ME might be a useful alternative to biopsies, especially for short-term follow-up after complete *en bloc* resection by ESD.

There were, however, some limitations to our study: (1) we focused on the simple characteristics of the mucosal pit structures of scar lesions at 2 mo after ER. We could not evaluate the vascular pattern and the validity of various pathologic findings using our short-term results. In addition, we need a long-term follow up study to confirm the final histology in lesions shown to be non-neoplastic in nature with type Ⅰ-Ⅲ pit patterns. (2) We enrolled only nine cases with type Ⅳ pit pattern because the therapeutic outcome of ER is excellent in gastric neoplasms. We could not discuss the diagnostic accuracy overall but could only do so in the nine cases with type Ⅳ pit pattern. A larger study with more cases to obtain a statistically meaningful accuracy is required in order to detect tumor recurrence in scar lesions following ER. (3) In terms of our procedure, the use of a transparent cap limited our survey capacity because it produced a narrow window of view and was very time consuming. After these procedural handicaps are overcome, large-scale and longitudinal follow-up studies should be pursued.

In conclusion, ME findings can detect the presence of tumors through detailed classification of post-ER scar lesions. ME may also help in decision-making regarding whether to perform biopsies and in identifying the target biopsy site in the short-term follow-up of post-ER scars in early gastric neoplasm. As stated above, however, further large-scale and long-term studies are required to determine whether ME can replace endoscopic biopsy.

COMMENTS

Background

Magnifying endoscopy (ME) is now being used in the diagnosis of various gastrointestinal diseases. However, not much data is currently available regarding the correlation between the findings on ME and pathological findings on post-endoscopic resection (ER) scars. There has been no definitive endoscopic description of which endoscopic findings require endoscopic biopsy or where the endoscopist should target the biopsy in altered large scar lesions.

Research frontiers

In this study, the authors demonstrate the relationship between the real-time diagnosis of post-ER scars observed using ME and the pathological diagnosis, thereby validating the clinical usefulness of ME as a follow-up method for post-ER scars in early gastric neoplasm.

Innovations and breakthroughs

This study gives the first description in the English literature of ME classification and the characteristic definition of gastric post-ER scar lesions. In addition, it includes comparative pathology for both the magnifying and conventional findings. Furthermore, our study suggests that ME can detect the presence of tumors through pit classification and may help in decision-making regarding the target biopsies in the short-term follow-up of post-ER scars.

Applications

By providing an understanding of how ME permits visualization of post-ER scars, this study may represent a future strategy in the short-term follow-up of post-ER scars in early gastric neoplasm.

Terminology

The mucosal pits, which were magnified up to 80 times with ME, were classified into four patterns of sulci and ridges: (Ⅰ) round pit patterns; (Ⅱ) short rod or tubular pit patterns; (Ⅲ) branched or gyrus-like pit patterns; and (Ⅳ) destroyed pit patterns. The criteria for suspecting a tumorous lesion included the observation of primarily a destroyed pit pattern.

Peer review

The authors investigated the pit patterns of post-ER scars using ME in early gastric neoplasm. It was revealed that all tumor lesions noted were in the type Ⅳ pit pattern. The results suggest that the ME pit patterns may be considered a useful diagnostic tool capable of replacing the more invasive endoscopic biopsy or of locating the target biopsy site in cases with mixed pit patterns, and may also help in the decision-making regarding whether to perform biopsies in the short-term follow-up of post-ER scars in early gastric neoplasm.

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BRIEF ARTICLES

Genomic-wide analysis of lymphatic metastasis-associated genes in human hepatocellular carcinoma

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Abstract

AIM: To identify the genes related to lymph node metastasis in human hepatocellular carcinoma (HCC), 32 HCC patients with or without lymph node metastasis were investigated by high-throughput microarray comprising 886 genes.

METHODS: The samples of cancerous and non-cancerous paired tissue were taken from 32 patients with HCC who underwent hepatectomy with lymph node dissection. Total RNA was extracted from the cells obtained by means of laser microdissection (LCM) and was amplified by the T7-based amplification system. Then, the amplified samples were applied in the cDNA microarray comprising of 886 genes.

RESULTS: The results demonstrated that 25 up-regulated genes such as cell membrane receptor, intracellular signaling and cell adhesion related genes, and 48 down-regulated genes such as intracellular signaling and cell cycle regulator-related genes, were correlated with lymph node metastasis in HCC. Amongst them were included some interesting genes, such as *MET, EPHA2, CCND1, MMP2, MMP13, CASP3, CDH1*, and *PTPN2*. Expression of 16 genes (*MET, CCND1, CCND2, VEGF, KRT18, RFC4, BIRC5, CDC6, MMP2, BCL2A1, CDH1, VIM, PDGFRA, PTPN2, SLC25A5* and *DSP*) were further confirmed by real-time quantitative reverse transcriptional polymerase chain reaction (RT-PCR).

CONCLUSION: Tumor metastasis is an important biological characteristic, which involves multiple genetic changes and cumulation. This genome-wide information contributes to an improved understanding of molecular alterations during lymph node metastasis in HCC. It may help clinicians to predict metastasis of lymph nodes and assist researchers in identifying novel therapeutic targets for metastatic HCC patients.

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Key words: Hepatocellular carcinoma; Lymphatic metastasis-associated genes; cDNA microarray; Expression profiling

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INTRODUCTION

Hepatocellular carcinoma (HCC), endemic to sub-Saharan African and Asian with a rising incidence in Western countries, is one of the most common fatal malignancies in the world[1-3]. This is due to different risk factors. Chronic hepatitis B virus (HBV), hepatitis C virus (HCV) infection[4] and exposure to the carcinogen aflatoxin[5] are important risk factors for African and Asian populations. HCC has a poor prognosis, with a 5-year survival of less than 3% in inoperable cases. The high mortality associated with this disease is mainly attributed to its high tendency to metastasize. In fact, local lymph node and blood metastases could occur at an early stage, which may be the key factors related to its recurrence and poor prognosis[5]. Thus, a better understanding of the molecular mechanism of metastasis can improve prevention and treatment of HCC.

Metastatic spread of tumor cells is a process involving multiple steps. To metastasize, tumor cells need to detach from the primary tumor mass, migrate to a distant secondary site, and rapidly expand in the new environment. The whole process requires activation and deactivation of multiple specific genes[6,7]. The present challenge is to identify the crucial genes controlling the metastasis and determine the regulatory mechanism of these genes. Hence, global analysis of expression profiles of a large number of genes in clinical HCC specimens is an essential step to clarify the detailed mechanism and discover potential biomarkers of lymphatic metastasis in HCC. Microarray techniques, which have been developed since the early 1990s, provide a platform where one can measure the expression levels of tens of thousands of genes in a sample simultaneously[8-10], and it is now possible to uncover the complete picture of lymphatic metastasis of HCC. In the current study, we analyzed gene expression profiles in a total of 32 HCC patients using cDNA microarray technology and their relation to pathological features based on lymph node metastasis staging. Validating the cellular functions of these genes will help to identify the key or candidate genes/pathways responsible for lymph node metastasis, which might be used as diagnostic markers and therapeutic targets for lymph node metastasis.

MATERIALS AND METHODS

Patient material

The Institutional Review Board on Medical Ethics, Zhejiang Provincial People Hospital (China), approved the method of tissue collection. The present study was based on 32 patients who underwent hepatectomy for sporadic HCC without preoperative radio- or chemotherapy in the Surgery Department, Zhejiang Provincial People Hospital. All of the samples were immediately frozen in liquid nitrogen, and stored at -80℃ until use. A total of 32 HCC samples from 15 lymph node-negative and 17 lymph node-positive cases were used (Table 1).

Table 1 Clincal data of patients with hepatocellular carcinoma

Case	Sex	Age	Hepatitis virus	Differentiated grade	TNM score
1	M	54	HBV	WD	T1N0M0
2	M	60	HCV	WD	T1N0M0
3	F	61	HBV	WD	T2N0M0
4	M	62	HBV	WD	T2N0M0
5	M	58	HBV	WD	T1N0M0
6	F	56	HCV	MD	T3N0M0
7	F	44	HBV	WD	T2N0M0
8	M	49	HCV	WD	T1N0M0
9	M	58	HBV	WD	T2N0M0
10	M	67	HCV	PD	T3N0M0
11	M	69	HBV	WD	T2N0M0
12	F	63	HCV	WD	T1N0M0
13	M	48	HCV	MD	T2N0M0
14	F	63	HBV	WD	T1N0M0
15	M	49	HCV	MD	T1N0M0
16	F	51	HBV	PD	T3N1M0
17	M	65	HCV	MD	T3N1M0
18	F	58	HBV	PD	T4N1M1
19	M	60	HBV	MD	T2N1M0
20	F	56	HCV	PD	T3N1M1
21	M	42	HCV	PD	T3N1M0
22	M	55	HBV	PD	T4N1M1
23	M	66	HBV	MD	T3N1M0
24	F	70	HCV	WD	T2N1M0
25	M	58	HBV	PD	T4N1M1
26	M	53	HCV	PD	T3N1M0
27	M	61	HBV	PD	T4N1M0
28	F	65	HBV	MD	T3N1M0
29	M	59	HCV	MD	T3N1M1
30	M	50	HBV	PD	T3N1M0
31	F	63	HCV	PD	T4N1M1
32	M	66	HCV	PD	T3N1M0

M: Male; F: Female; HBV: Hepatitis B virus infection; HCV: Hepatitis C virus infection; WD: Well differentiated HCC; MD: Moderately differentiated HCC; PD: Poorly differentiated HCC.

Laser microdissection

The 8 μm-thick sections of frozen tissue were continuously cut at -20℃ and stained with H&E. Under microscopic observation, parts of cancer cell nests in the invasive and intraductal components were microdissected using the LM100 laser capture microdissection system (Arcturus Engineering, Mountain View, CA, USA). We used a 15 μm-diameter beam to capture the tumor cells and corresponding noncancerous liver tissues, respectively. The cell nests were transferred to the laser microdissection (LCM) transfer film (CapSure TF-100S transfer film carrier, 5 mm-diameter optical-grade transparent plastic; Arcturus Engineering).

RNA preparation and T7-based RNA amplification

Total RNA was isolated from the dissected specimens using Trizol reagent (Gibco BRL) and a modified acidic guanidinium phenol-chloroform method, following the manufactures recommendations. Total RNA was treated with DNase I for removal of genomic DNA. mRNA was purified using a poly(A) purification kit (Oligotex, Qiagen) according to the manufactures instructions. The quality of mRNA was assessed by OD 260/280 ratios and the contamination of genomic DNA was checked using the PCR method. cDNA was synthesized with T7-oligo (dT) primer (Ambion) and Superscript Ⅱ enzyme (Gibco BRL) following

the instruction manual. cDNA was purified by cDNA clean-up column (DNA clear™ kit, Ambion). cRNA was generated by T7 MEGAscript™ kit (MEGAscript *in vitro* Transcription Kit, Ambion, Austin, TX, USA) following the manufactures recommendations. Column purification of cRNA was performed with RNeasy kit (Qiagen) according to the manufactures protocol. The concentration and quality of cRNA were analyzed by GeneQuant pro RNA/DNA Calculator (Amersham Pharmacia Biotech, Buckinghamshire, England).

Microarray hybridization and scanning

Human Cancer Chip version 4.0 (IntelliGene, TaKaRa) was used for these studies. This array was spotted with 886 cDNA fragments of human genes, which are composed of 588 kinds of human identified genes related to cancer and 298 cDNA fragments prescreened by differential display methods between cancer tissue and normal tissues, on a glass slide. Three μg of cRNA from the tumor and the matched normal tissue were respectively labeled with Cy3-dUTP and Cy5-dUTP (Amersham Pharmacia Biotech, Buckinghamshire, England) using a labeling kit (RNA Fluorescence Labeling Core kit, TaKaRa), following the manufactures instructions. Labeled probe was purified by centrifugation in a spin column (Centrisep, Princeton Separations, Adelphia, NJ). Two separate probes were combined, and then, 2 μL of 5 × competitor containing CotI (Gibco BRL), poly dA (Amersham Pharmaca Biotech), and tRNA (TaKaRa) were added. After addition of 50 μL of 100% ethanol and 2 μL of 3 mmol/L sodium acetate (pH 5.2), the mixture was cooled at -80℃ for 30 min, followed by centrifugation at 15000 rpm for 10 min. For final probe preparation, the pellet was washed in 500 μL of 70% ethanol twice, and eluted in 10 μL hybridization buffer (6 × SSC, 0.2% SDS, 5 × Denhardt's solution, 0.1 mg/mL salmon sperm solution). The probes were denatured by heating for 2 min at 95℃, cooled at room temperature, and centrifuged at 15000 rpm for 10 min (20-26℃). Supernatants were placed on the array and covered with a 22 mm × 22 mm glass coverslip. The coverslip was sealed with a glue, and the probes were incubated overnight at 65℃ for 16 h in a custom-made slide chamber with humidity maintained by underlying moist papers. After hybridization, the slides were washed in 2 × SSC with 0.1% SDS, 1 × SSC, and 0.05 × SSC, sequentially for 1 min each, and then spin dried. Hybridized arrays were scanned using a confocal laser-scanning microscope (Affymetrix 428 array scanner, Santa Clara, CA). Image analysis and quantification were performed with ImaGene 4.2 software (BioDiscovery) as per the manufactures instructions.

Data processing

Each spot was defined by manual positioning of a grid of circles over the array image. For each fluorescent image, the average pixel intensity within each circle was determined, and a local background outside of 3 pixel buffer range from the circle was computed for each spot. Net signal intensity was determined by subtraction of this local background from the average intensity of each spot. Signal intensities between the two fluorescent images were normalized by the intensities of the housekeeping genes provided on the arrays. The fluorescence intensities of Cy5 (non-tumor) and Cy3 (tumor) for each target spot were adjusted so that the mean Cy3:Cy5 ratios of 32 housekeeping gene spots were equal to one. Because data derived from low signal intensities are less reliable, we first determined cutoff values for signal intensities on each slide so that all of the filtered genes had greater S:N (signal to noise) ratios of Cy3 or Cy5 than three, and we excluded genes for further analysis when both Cy3 and Cy5 dyes gave signal intensities lower than the cutoff. To estimate the range of expression ratio within which the expression change could be considered as fluctuation in noncancerous cells, we compared expression profiles of noncancerous cells from 6 patients. Because 90% of expression ratios in noncancerous cells fell within the range of 1.726 and 0.503, we categorized genes into three groups according to their expression ratios (Cy3:Cy5): up-regulated (ratio: 2.0); down-regulated (ratio: 0.5); and unchanged expression (ratios between 0.5 and 2.0); provided that signal counts of T (Cy3) and R (Cy5) were > 500. Genes with Cy3:Cy5 ratios > 2.0 or < 0.5 in more than 75% of the cases examined were defined as commonly up- or down-regulated genes, respectively.

Real-time reverse transcription-PCR

LightCycler (Roche Diagnostics) technology was applied to confirm the data which were obtained by cDNA microarray. The primer sequences of 16 genes were obtained from the GDB Human Genome Database (http://www.gdb.org/gdb/). We used the same RNA from the dissected cells in microarray analysis. First-strand cDNA was obtained by reverse transcription using a commercially available kit (first strand synthesis kit, Amersham). For each PCR, 2 μL (20 ng) first strand cDNA template, 50 pmol of each primer, 2.4 μL (3 mmol/L) $MgCl_2$, and 2 μL 10 × SYBR Green I (Roche Laboratories) were mixed in 20 μL of PCR mixture. The running protocol has been programmed based on the following three steps. In the first step, initial denaturation, reaction mixture was incubated for 10 min at 95℃. In the second step, DNA was amplified for 45 cycles at 95℃ for 10 s, specific annealing temperature (the primer sequences dependent) for 0-10 s, and elongation at 72℃ for some seconds (amplicon [bp]/25 s). Finally, the temperature was raised gradually (0.2℃/s) from the annealing temperature to 95℃ for the melting curve analysis. Twelve μL of PCR products were visualized by electrophoresis on 2% agarose gel stained with ethidium bromide. The amount of gene expression was normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using Human GAPDH kit (GmbH Heidelberg, Heidelberg, Germany). We carried out qRT-PCR analysis in triplicate for each cDNA sample and used median values in three experiments. Up- and down-regulation were defined as the median value > 2.0 and < 0.5, respectively.

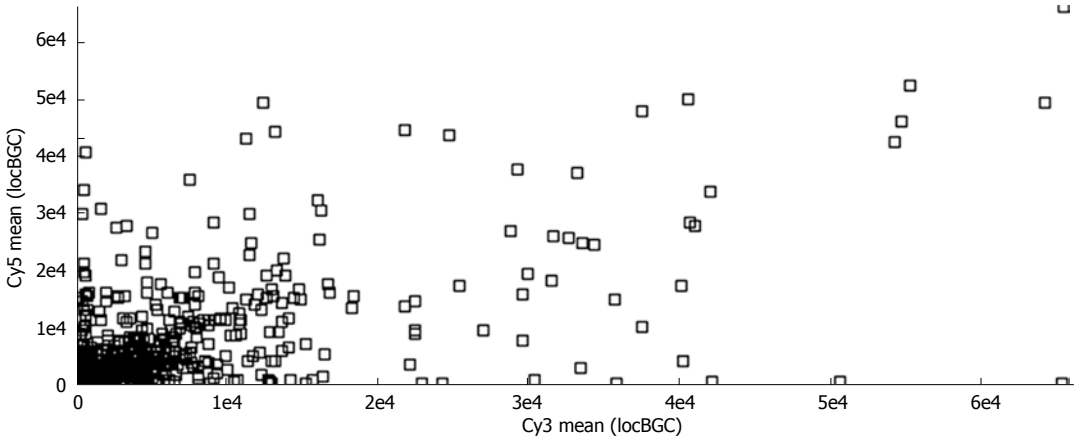

Figure 1 Scatter plots of cDNA microarray analysis. Primary carcinoma cells (Cy3-labeled) and normal cells (Cy5-labeled) from case 20 are labeled and hybridized to the cDNA microarray.

Statistical analysis

A statistical analysis among mean values was performed on the association of lymph node metastasis with expression levels by applying non-parametric Kruskal-Wallis and Mann-Whitney *U* tests. Statistical significance was defined as a *P*-value < 0.05. Differential expression between the groups of lymph node metastasis and the group of non-lymph metastasis was considered significant, where *P* value < 0.05.

RESULTS

Quality analysis of total RNA after LCM and cRNA after T7-based amplification was carried out. About 20 slides were prepared in every sample, and the target cells were captured with at least approximately 1000 cells per slide. Consequently, we captured a total of approximately 25000-30000 tumor and normal cells for RNA extractions, respectively. The quality of total RNA extracted after LCM was assessed by A260/A280 and electrophoresis. To be considered for microarray analysis, the RNA samples needed to pass the quality control criteria, namely integrity of 28S and 18S, and A260/A280 greater than 2.0. Products of cDNA synthesis and cRNA were also checked by A260/A280 and electrophoresis. Results showed that A260/A280 of all the RNA samples met the quality control criteria for sample preparation. Clear image appearance of 28S and 18S of ribosomal RNA was seen under the electropherogram for each total RNA sample, which had to be considered as intact or without degradation. RNA was subjected to two rounds of T7-based RNA amplification after removal of DNA contamination by RNase-free DNase I treatment as described in Methods. All RNA was successfully amplified an estimated 250-fold by using T7 RNA polymerase. cDNA synthesis and cRNA showed satisfactory quality control criteria, which was 1.5 kb < cDNA < 5.0 kb; 1.0 kb < cRNA < 4.5 kb); and A260/A280 ratio of cDNA and cRNA greater than 2, respectively.

Identification of expressed genes associated with lymph node metastasis

After reverse transcription, each cDNA probe was labeled with Cy3- or Cy5-conjugated dyes and hybridized to microarray cDNAs with 886 genes. We evaluated the expression profiles comparing the cancer cells and the corresponding normal cells in each case. A representative scatter plot of microarray analysis between the metastatic carcinoma cells and non-cancerous tissue in case 20 is shown in Figure 1. Up-, down-regulated and unchanged genes indicated by red, green and blue spots respectively are shown in Figure 2. We first arranged the relative expression of each gene (Cy3/Cy5 intensity ratio) into one of four categories: up-regulated (ratio: > 2.0), down-regulated (ratio: < 0.5), unchanged (ratio: between 0.5 and 2.0), and not expressed (or slight expression but under the cutoff level for detection).

To identify the genes related to lymph node metastasis, 32 cases were divided into two groups: a metastatic group in which lymph node metastasis was positive in 17 patients (No. 16-32) and a non-metastatic group in which lymph node metastasis was negative in 15 patients (No. 1-15) (Table 1). When comparing gene expression profiles between two groups, there were 25 genes that were commonly up-regulated and expressed more than 1.87-fold in the lymphatic metastasis groups compared with those in the negative groups. On the other hand, 48 down-regulated expressed genes were significantly correlated with the lymphatic metastasis groups. Tables 2 and 3 show the list of these differentially expressed genes and their category based on GO (Gene Ontology) system and TreeView. The up-regulated genes were associated with cell adhesion molecules, cell membrane receptors, intracellular signaling related genes, etc. The up-regulated genes included interesting genes, such as *MET*, *EPHA2*, *CCND1*, *MMP2* and *MMP13*. The down-regulated genes were mostly cell adhesion molecules, cell cycle regulators and intracellular signaling molecules. The down-regulated genes included *CASP3*, *CDH1*, and *PTPN2*.

Gene expression confirmation by real-time RT-PCR

To investigate the reliability of cDNA microarray data, real-time quantitative RT-PCR was performed for measuring the expression levels of 16 genes (*MET*, *CCND1*, *CCND2*, *VEGF*, *KRT18*, *RFC4*, *BIRC5*, *CDC6*,

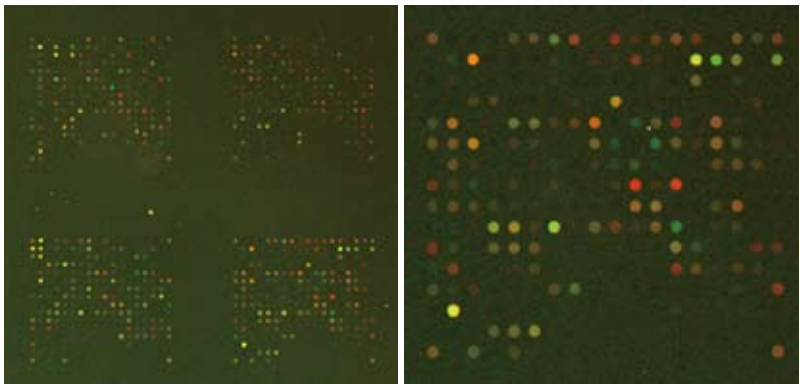

Figure 2 A representative of cDNA microarray expression pattern obtained from case 20. Up-, down-regulated and unchanged genes are indicated by red, green and blue spots, respectively.

Table 2 Up-regulated genes correlated with lymphatic metastasis

Gene name	Symbol[a]	Accession[b]	Fold change[c]	pN1:pN0[d]
Cell adhesion proteins				
CD58 antigen, (lymphocyte function-assocoated antigens)	CD58	NM_001779	7.75	2.28
Integrin αM	ITGAM	NM_000632	5.04	2.06
Integrin β5	ITGB5	NM_002213	3.98	1.87
Opioid-binding protein/cell adhesion molecule-like	OPCML	NM_002545		
Cell membrane receptor				
CD86 antigen, (CD28 antigen ligand 2, B7-2 antigen)	CD86	NM_006889	6.76	2.33
v-jun sarcoma virus 17 oncogene homolog (avian)	JUN	NM_002228	7.43	2.27
Met proto-oncogene (hepatocyte growth factor receptor)	MET	NM_000245	10.11	3.46
EphA2	EPHA2	NM_004431	8.26	2.13
Epidermal growth factor receptor [avian erythroblastic leukemia viral (v-erb-b) oncogene homolog]	EGFR	NM_005228	8.35	2.62
Cell death regulator				
BCL2/adenovirus E1B 19kDa interacing protein 3	BNIP3	NM_004052	3.88	2.29
Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D	SEMA4D	NM_006378	5.71	3.64
Intracellular signaling				
Rho GDP dissociation inhibitor γ	ARHGDIG	NM_001175	5.37	1.97
Ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3)	RAC3	AK054993	4.63	2.07
Insulin-like growth factor binding protein 3	IGFBP3	M35878	5.39	2.15
Coagulation factor Ⅱ (thrombin) receptor	F2R	NM_001992	6.17	2.43
Growth/differentiation factor				
Vascular endothelial growth factor C	VEGFC	NM_005429	5.68	2.47
Cell cycle regulator				
Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	CCND1	NM_053056	11.56	4.31
Cyclin-dependent kinase 4	CDK4	NM_000075	8.73	3.59
Others				
Tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	TIMP3	NM_000362	3.95	1.89
Ubiquitin-conjugating enzyme E2A (RAD6 homolog)	UBE2A	NM_003336	4.38	1.95
v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	YES1	NM_005433	5.66	2.37
P450(cytochrome) oxidoreductase	POR	AF258341	4.74	1.99
Matrix metalloproteinase 13	MMP13	NM_00247	5.67	2.45
Matrix metalloproteinase 2 (gelatinase A, 72kD gelatinase, 72kD type Ⅳ collagenase)	MMP2	NM_004530	7.13	3.36

[a]Symbol in LocusLink database; [b]GeneBank accession number; [c]Fold change, ratio of mean expression values in lymph node metastasis cases (cancer cells *vs* non-cancerous cells); [d]pN1:pN0, ratio of mean expression values (lymph node positive cases to lymph node negative cases).

MMP2, *BCL2A1*, *CDH1*, *VIM*, *PDGFRA*, *PTPN2*, *SLC25A5* and *DSP*). One representative case (case 8) is shown in Figure 3. We used cDNA synthesized from 32 pair samples without amplification as template for real-time quantitative reverse transcription PCR. The results demonstrated that the samples obtained by means of T7-based amplification well reflected the status of the original RNA in a proportional manner.

DISCUSSION

The application of high-throughput cDNA microarray permits simultaneous analysis of genome-wide expression of thousands of genes in a sample and to investigate the correlation between clinicopathological phenotypes and gene expression status[8-10]. This technology is a powerful tool for screening genes,

Table 3 Down-regulated genes correlated with lymphatic metastasis

Gene name	Symbol[a]	Accession[b]	Fold change[c]	pN1:pN0[d]
Cell adhesion proteins				
Desmoplakin (DPI, DPII)	DSP	NM_004415	-14.22	0.24
Protocadherin gamma subfamily C, 3	PCDHGC3	NM_002588	-7.88	0.41
Integrin, beta 4	ITGB4	NM_000213	-13.39	0.26
Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	ITGA3	NM_002204	-6.24	0.38
Catenin (cadherin-associated protein), alpha 1 (102kD)	CTNNA1	NM_001903	-8.53	0.37
Cadherin 1, type 1, E-cadherin (epithelial)	CDH1	NM_004360	-15.85	0.25
Cell cycle regulator				
Cell division cycle 25B	CDC25B	NM_021784	-6.90	0.46
Cyclin-dependent kinase 5	CDK5	NM_004935	-5.51	0.48
Cyclin D2	CCND2	NM_001759	-6.29	0.43
Microtubule-associated protein, RP/EB family, member 1	MAPRE1	NM_012325	-13.16	0.29
Protein phosphatase 1D	PPM1D	NM_003620	-11.77	0.39
Ataxia telangiectasia and Rad3 related	ATR	NM_001184	-7.86	0.41
Protein phosphatase 2, regulatory subunit B (B56), alpha isoform	PPP2R5A	NM_006243	-3.89	0.49
Intracellular signaling				
Mitogen-activated protein kinase 14	MAPK14	NM_001315	-5.67	0.43
Mitogen-activated protein kinase 7	MAPK7	NM_002749	-7.15	0.39
Mitogen-activated protein kinase 4	MAPK4	NM_002747	-3.83	0.32
Small inducible cytokine B subfamily (Cys-X-Cys motif), member 13 (B-cell chemoattractant)	SCYB13	NM_006419	-4.58	0.46
Signal transducer and activator of transcription 5B	STAT5B	NM_012448	-8.13	0.37
Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	SERPINB1	NM_030666	-4.47	0.48
Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	SERPINB2	NM_002575	-5.72	0.41
SH3-domain binding protein 2	SH3BP2	AB000462	-3.29	0.52
G protein-coupled receptor kinase 6	GPRK6	NM_002082	-14.12	0.29
GTP-binding protein ragB	RAGB	NM_016656	-6.77	0.46
Protein tyrosine phosphatase, receptor type, F	PTPRF	NM_002840	-7.21	0.34
Cell membrane receptor				
EphB6	EPHB6	NM_004445	-12.88	0.38
Kangai 1 (suppression of tumorigenicity 6, prostate; CD82 antigen (R2 leukocyte antigen, antigen detected by monoclonal and antibody IA4)	KAI1	NM_002231	-2.24	0.67
Small inducible cytokine subfamily A (Cys-Cys), member 25	SCYA25	NM_005624	-3.76	0.72
Monoglyceride lipase	MGLL	NM_007283	-4.66	0.43
Interferon (alpha, beta and omega) receptor 1	IFNAR1	NM_000629	-2.39	0.81
Insulin-like growth factor binding protein 6	IGFBP6	NM_002178	-3.53	0.62
Metabolic enzyme				
Serine protease inhibitor, Kunitz type, 2	SPINT2	NM_021102	-5.44	0.46
Protein phosphatase 2, regulatory subunit B (B56), alpha isoform	PPP2R5A	NM_006243	-7.12	0.37
Protein tyrosine phosphatase, non-receptor type 2	PTPN2	NM_002828	-19.73	0.23
Deoxyribonuclease I-like 3	DNASE1L3	NM_004944	-8.23	0.29
Cathepsin L	CTSL	NM_001912	-4.39	0.37
Cell death regulator				
Caspase 3, apoptosis-related cysteine protease	CASP3	NM_004346	-14.57	0.29
Programmed cell death 10	PDCD10	NM_007217	-7.19	0.36
DNA damage response				
Ataxia telangiectasia and Rad3 related	ATR	NM_001184	-4.78	0.48
X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining; Ku autoantigen, 80kD)	XRCC5	NM_021141	-2.59	0.73
Mouse double min 2	MDM2	NM_002392	-6.37	0.51
Growth/differentiation factor				
Bone morphogenetic protein 5	BMP5	NM_021073	-2.78	0.53
Keratin 4	KRT4	NM_002272	-3.12	0.62
Keratin 13	KRT13	NM_002274	-4.24	0.48
Connective tissue growth factor	CTGF	NM_001901	-5.51	0.42
Others				
Heat shock 70kDa protein 4	HSPA4	AB023420	-3.19	0.56
Retinoid X receptor, α	RXRA	NM_002957	-2.89	0.77
Ubiquitin-activating enzyme E1-like	UBE1L	NM_003335	-2.95	0.53
Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	SLC25A5	NM_001152	-6.18	0.46

[a]Symbol in LocusLink database; [b]GeneBank accession number; [c]Fold change, ratio of mean expression values in lymph node metastasis cases (cancer cells *vs* non-cancerous cells); [d]pN1:pN0, ratio of mean expression values (lymph node positive cases to lymph node negative cases).

the expression of which can be correlated with pathological phenotypes of various tumors[11,12]. Analysis of gene expression profiles not only has disclosed specific patterns that may reflect prognosis and drug sensitivity of tumor cells but has also revealed the identity of genes involved in malignant transformation,

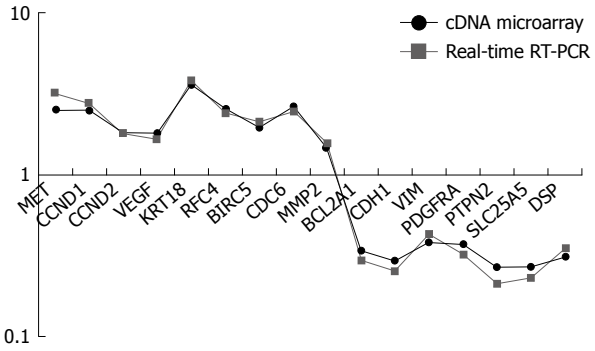

Figure 3 Relative abundance of the mRNA levels of 16 genes in one representative case (case 8), determined by cDNA microarray and real-time RT-PCR, respectively.

progression, and metastasis of tumors[13-16]. However, the existence of bulky surrounding cells produces much interferential noise information because of interstitial effects[17]. Therefore, selection of cancer cells using LCM is of indispensable value in combination with the cDNA microarray. This study clearly demonstrated that an analysis of global gene expression profiles can be performed with RNA samples obtained from primary HCC tissues by using LCM, T7-based RNA amplification, and cDNA microarray. It is thus possible to focus directly on the gene expression profile of an individual cell population consisting of tumor tissue.

Lymph node metastasis is one of the most important prognostic factors in HCC patients[18,19]. This is a highly selective sequential step involving multiple genes, multiple signals pathways and regulatory mechanisms during the process, which favors the survival of a subpopulation of metastatic cells preexisting within the primary tumor mass to produce clinically relevant metastases[20-23]. The metastatic cells exhibit a complex phenotype that is regulated by transient or permanent alterations in various genes at mRNA level. In the present study of HCC patients, we compared gene expression in lymph node metastasis and in those without lymph node metastasis by using cDNA microarray analysis. We found that 25 up-regulated and 48 down-regulated genes were correlated with lymph node metastasis of HCC. The up-regulated genes included cell adhesion related genes (*CD58*, *ITGAM*, *ITGB5*, *OPCML*), cell membrane receptor (*CD86*, *JUN*, *MET*, *EPHA2*, *EGFR*), cell death regulator (*BNIP3*, *SEMA4D*), intracellular signaling related genes (*ARHGDIG*, *RAC3*, *IGFBP3*, *F2R*), growth/differentiation factor (*VEGFC*), cell cycle regulator (*CCND1*, *CDK4*), and other genes (*TIMP3*, *UBE2A*, *YES1*, *POR*, *MMP13*, *MMP2*). The down-regulated genes included cell adhesion related genes (*DSP*, *PCDHGC3*, *ITGB4*, *ITGA3*, *CTNNA1*, *CDH1*), cell cycle regulator (*CDC25B*, *CDK5*, *CCND2*, *MAPRE1*, *PPM1D*, *ATR*, *PPP2R5A*), intracellular signaling related genes (*MAPK14*, *MAPK7*, *MAPK4*, *SCYB13*, *STAT5B*, *SERPINB1*, *SERPINB2*, *SH3BP2*, *GPRK6*, *RAGB*, *PTPRF*), cell membrane receptor (*EPHB6*, *KAI1*, *SCYA25*, *MGLL*, *IFNAR1*, *IGFBP6*), metabolic enzyme (*SPINT2*, *PPP2R5A*, *PTPN2*, *DNASE1L3*, *CTSL*), cell death regulator (*CASP3*, *PDCD10*), DNA damage response related genes (*ATR*, *XRCC5*, *MDM2*), growth/differentiation factor (*BMP5*, *KRT4*, *KRT13*, *CTGF*), and other genes (*HSPA4*, *RXRA*, *UBE1L*, *SLC25A5*).

Amongst these genes are some which are well documented in the literature as being involved in the malignant potential of some types of carcinomas. CCND1, one of the cell cycle regulators, is synthesized in the G1 phase, mixed with CDK2, CDK4 and CDK5, and related closely with cell cycle control[24,25]. Our results showed that CCND1 is highly expressed in HCC with lymphatic metastasis, which may be a useful marker that relates with the poor prognosis of HCC, and overexpression of CCND1 in HCC without lymphatic metastasis may be used as useful information for treatment measures after operation[26,27]. CDK4, an important member of the CDKs protein family, was correlated positively with CCND1 as reported by some documents[24-26]. Our results showed that CDK4 was remarkably up-regulated in HCC patients with lymph node metastasis, and was consistently correlated with that of CCND1[28]. EphA2, one member of the tyrosine activating enzyme acceptor Eph family, can be used as a ligand of ephrins connecting with cells and involved in cell interaction[29]. Our study demonstrated that up-regulation of EphA2 was related to lymph node metastasis of HCC, however, the mechanism remains in need of further studies. It has been proven that the formation of new blood vessels plays a role in the growth and metastasis of solid tumors, and various growth factors secreted from tumor cells determine the pace of the progression process[30]. It is documented that VEGF is up-regulated in most solid tumors, whereas there is few or none in the normal tissues, and its expression level is positively related with microvessel density, invasion, metastasis and prognosis of tumors[31]. Present results showed that the expression levels of VEGF were related closely to the clinical stage of HCC, its expression levels increased gradually with the increase of TNM, there were significant differences in expression levels between HCC patients with and those without lymph node metastasis ($P < 0.05$), and its expression levels were a useful marker of the recurrence, the distant metastasis and the poor prognosis of HCC (data not shown). The extracellular matrix (ECM) is the first barrier during the process of invasion and metastasis of tumor cells[32]. Tumor cells and their neighbor interstitial cells, such as the endothelial cell, the macrophage and so on, may produce a great quantity of proteinase for degradation of ECM, and help tumor cells to migrate easily. Of these proteinases, the matrix metalloproteinase (MMP) is an important one. MMPs can degrade the stroma collage, which favors the action of tumor cells to shake off the yoke of ECM and to migrate. This is the biochemical basis of the circumambience invasion of tumor cells[33,34]. It was found that MMP13 and MMP2 expressions in HCC with lymph node metastasis were significantly higher than that of those without lymph node metastasis, and MMP2 in the lymph

node metastasis group was 3.12-fold of those without metastasis. Lymph node metastasis of HCC may be related to the "thundering" activity of the growth factor signaling pathway[35]. It has been proven that the signaling pathway of tyrosine kinase receptor and G-protein connective receptor are the most important two of all pathways[36]. EGFR, containing the sequence of tyrosine kinase, was always up-regulated in HCC with lymph node metatasis patients. It has been reported that the signaling pathway mediated by all growth factors for cell proliferation purposes was always dependent on the tyrosine kinase receptor signaling pathway, and tyrosine kinase was kept activated in many tumors[37].

It has been reported that down-regulation of desmoplakin (DSP, member of the cadherin family) is correlated with tumor invasion and metastasis[38]. Our results showed that DSP was suppressed in the lymph node metastasis group compared with the non-metastasis group. Tumor cells always secrete some new adhesion molecules when removed from the primary lesion and grown again in another position, whereas CTNNA 1 was down-regulated in our results, suggesting its role was maybe different from that of the primary lesion[39,40]. E-cadherin (a cell membrane superficial molecule mediating adhesion among normal cells) was down-regulated in lymph node metastasis HCC patients. Ephrin, ligand of tyrosine kinase receptor, was also down-regulated in the metastasis group. It was reported that the combination of ephrin-B2 ligand and EphB4 was related to the occurrence and metastasis of some solid tumors[41,42]. KAI1, a new gene found recently, was down-regulated in many lymphatic metastasis tumors, and this gene is associated with the motion and metastasis of tumor cells[43]. Protein tyrosine phosphatase, which has an action contrary to that of tyrosine kinase, takes part in signal regulation, energy metabolism, cell proliferation and promoting MHCI expression mediated by many hormones, such as insulin and epidermal growth factor, and others[44,45]. The decrease of tyrosine phosphatase activity may reduce the MHCI expression of cells superficially so that tumor cells escape the inspection of the immunological system[44]. PTPN2 and PTPRF were down-regulated in the lymph node metastasis group, suggesting that the tyrosine phosphatase was associated with invasion and metastasis of tumor cells[44-46].

Our study demonstrated that lymph node metastasis comes from the result of the structural and functional abnormality of cellular and extracellular multigenes, since many genes and signaling pathways play key roles during the metastasis of tumor cells by dominating the cell proliferation, differentiation and death. Potential metastatic biological behavior of tumor cells was characteristically the release of cell-cell adhesion, the abnormality of cell cycle regulator and cell signal pathways, which suggest that the invasive character of tumor cells is determined by cellular interaction with the extracellular environment rather than the proliferative potential. The abnormality of apoptosis and proliferation ability may occur owing to loss of control of the cell cycle and the obstruction of cell signal molecules transmitting communication, which may be is one of the mechanisms of accelerating tumor invasion and metastasis[47,48]. Although we were able to extract some genes related to lymph node metastasis in HCC, further examination is necessary of other genes as well as the interaction with stromal tissues. Since these genes are thought to affect each other, it is important to further analyze each gene in detail to elucidate the mechanism of lymph node metastasis in HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC), endemic to sub-Saharan Africa and Asia with a rising incidence in Western countries, is one of the most common fatal malignancies in the world. The high mortality associated with this disease is mainly attributed to its high tendency to metastasize. In fact, local lymph node and blood metastases could occur at an early stage, which may be the key factors relating to its recurrence and poor prognosis. Thus, a better understanding of the molecular mechanism of metastasis can improve prevention and treatment of HCC.

Research frontiers

The purpose of this study is to identify the genes related to lymph node metastasis in human hepatocellular carcinoma (HCC). Thirty two HCC patients with or without lymph node metastases were investigated by high-throughput microarray comprising 886 genes. The results demonstrated that 25 up-regulated genes such as cell membrane receptor, intracellular signaling and cell adhesion related genes, and 48 down-regulated genes such as intracellular signaling and cell cycle regulator-related genes, were correlated with lymph node metastasis in HCC.

Innovations and breakthroughs

The application of high-throughput cDNA microarray permits analysis of genome-wide expression of thousands of genes in a sample and thus to investigate the correlation between clinicopathological phenotypes and gene expression status. This study clearly demonstrated that an analysis of global gene expression profiles can be performed with RNA samples obtained from primary HCC tissues by using LCM, T7-based RNA amplification, and cDNA microarray. Thus, it is possible to focus directly on the gene expression profile of an individual cell population consisting of tumor tissue.

Applications

This genome-wide information contributes to an improved understanding of molecular alterations during lymph node metastasis in HCC. It may help clinicians to predict metastasis of lymph nodes and assist researchers in identifying novel therapeutic targets for metastatic HCC patients.

Terminology

DNA microarray is a high-throughput and powerful technology used in molecular biology and in biomedicine areas. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides. A short section of a gene or other DNA element can be used as a probe to hybridize a cDNA or cRNA sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by fluorescence-based detection of fluorophore-labeled targets to determine relative abundance of nucleic acid sequences in the target.

Peer review

The authors claimed that the expression of distinct sets of genes was either enhanced or decreased in hepatocellular carcinoma tissues with lymph node metastasis, compared with those without any lymph node metastasis. Based on the clinical information described in this article, the patients with lymph node metastasis are in more advanced stages (T3, T4, or M1), compared with those without metastasis. Thus, their observed changes in gene expression may arise from the local tumor growth or distant metastasis. The authors should exclude this possibility.

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BRIEF ARTICLES

Prophylactic effect of glyceryl trinitrate on post-endoscopic retrograde cholangiopancreatography pancreatitis: A randomized placebo-controlled trial

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Abstract

AIM: To examine the prophylactic effect of glyceryl trinitrate on post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis and hyperamylasemia.

METHODS: Patients scheduled for ERCP were randomly divided into study group and placebo group. Patients in study group and placebo group were treated with 5 mg glyceryl trinitrate and 100 mg vitamin C, respectively, 5 min before endoscopic maneuvers.

RESULTS: A total of 74 patients were enrolled in the final analysis. Post-ERCP pancreatitis occurred in 3 patients (7.9%) of the study group and 9 patients (25%) in the placebo group (P = 0.012). Hyperamylasemia occurred in 8 patients of the study group (21.1%) and 13 patients (36.1%) of the placebo group (P = 0.037).

CONCLUSION: Glyceryl trinitrate before ERCP can effectively prevent post-ERCP and hyperamylasemia.

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Key words: Glyceryl trinitrate; Cholangiopancreatography; Endoscopic retrograde; Pancreatitis

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is a widely applied method in the diagnosis and treatment of pancreatobiliary disease. Post-ERCP pancreatitis is the most common postoperative complication of ERCP. Although most cases of post-ERCP pancreatitis are mild, some may be severe and lethal. The incidence of post-ERCP pancreatitis is 1%-40%[1-3] and how to prevent it becomes an urgent clinical challenge. Some studies on drugs for preventing post-ERCP pancreatitis are available[4,5], but their results remain debatable. Therefore, most endoscopy centers do not give patients a conventional preventive drug therapy. Glyceryl trinitrate, a strong smooth muscle relaxant, is widely used in treatment of cardiovascular diseases. Glyceryl trinitrate could lower the basal pressure in the sphincter of Oddi and depress the resistance of bile outflow. Moretó *et al*[6] demonstrated that glycery trinitrate can reduce the incidence of post-ERCP pancreatitis. This prospective placebo-controlled double-blind randomized trial enrolled 74 patients scheduled for ERCP and observed the preventive effect of glycery trinitrate on post-ERCP pancreatitis.

MATERIALS AND METHODS

Study population

Seventy-four eligible patients at the age of 18 years and over were included in this study. ERCP was performed for them by the same experienced endoscopist.

Patients with acute or active chronic pancreatitis,

and a nitrate allergic history, and those undergone sphincterotomy, were excluded.

Research regimen

All the enrolled patients were randomly divided into study group and placebo group. Patients in study group took 5 mg sublingual glyceryl trinitrate 5 min before the procedure, while patients in placebo group took 100 mg sublingual vitamin C. Patients could receive antibiotics, analgesics or ataractics as needed, but somatostatin or octreotide was forbidden. Patients, operators or result observers were blinded to their grouping.

Observing targets

Serum amylase concentration in each patient was measured before and 4 and 24 h after endoscopy. Abdominal pain, fever, vomiting or other symptoms or signs were observed, and their laboratory or specifically evaluated results were recorded. Meanwhile, details of therapeutic endoscopic procedure, including expansion of bile duct, operating time (hours) and treatment, were also recorded.

Diagnostic criteria

According to the postoperative complications of ERCP[7,8], post-ERCP pancreatitis could be defined as a disease with sustained pancreatitis symptoms (such as abdominal pain) and high-amylase value over the normal value after ERCP. Hyperlipidemia was defined as the higher serum amylase concentration without or only with mild abdominal pain.

Statistical analysis

Data were analyzed using SPSS11.5 for statistics. Statistical analysis was performed by Student's *t*-test and χ^2-test.

RESULTS

General results

A total of 74 patients were randomly divided into study group (n = 36) and placebo group (n = 36). Of these patients, 6 were eliminated because of intubation failure, 1 had a BillrothII gastroectomy history, 2 did not allow endoscopy because of obstruction at duodenal descending part, and 3 failed to intubate the papilla. All the patients completed the trial. No significant difference was found in baseline characteristics between the two groups, such as gender, age, etiology, duct expansion, ERCP operating time, or treatment (Table 1).

Incidence of pancreatitis after ERCP

Post-ERCP pancreatitis occurred in 3 patients of the study group (7.9%), in 9 patients of the placebo group (25%), showing a significant difference between the two groups (P = 0.012). The condition of patients who developed post-ERCP pancreatitis was significantly improved after conservative treatment (Table 2).

Table 1 Baseline characteristics of study and placebo groups

	Study group	Placebo group	*P*
Demographic characteristics			
Number	38	36	
Sex ratio (M/F)	15/23	16/20	0.665
Mean age (yr)	64.29 ± 13.40	63.36 ± 15.13	0.781
Etiology			0.972
Choledocholithiasis (cases)	33	31	
Others(cases)	5	5	
Cholangiectasis (cases)	26	20	0.254
Treatment			0.841
Choledochostomy	26	26	
Stent intervention	6	6	
Sphincterotomy and drainage	6	4	
ERCP operating time (min)	36.89 ± 20.51	40.00 ± 24.73	0.558

Table 2 Complications occurred in study and placebo groups

Group	PEP	Hyperamylasemia	Normal
Study	3	8	27
Placebo	9	13	14
P	0.012	0.037	

Incidence of hyperamylasemia after ERCP

Hyperamylasemia occurred in 13 patients of the placebo group (36.1%) and 8 patients of the study group (21.1%). There was a significant difference between the two groups (P = 0.037, Table 2).

DISCUSSION

ERCP is an indispensable method for diagnosis and treatment of hepatic and pancreatobiliary disease. Pancreatitis is the most common postoperative complication of it. The nosogenesis may include[9]: (1) papilla edema due to reiterative intubation at duodenal papilla leading to pancreatic outflow obstruction, (2) pancreatic secretion caused by contrast agent over filling pancreatic duct or excessive contrast agent or bubbles entering the pancreas, (3) mechanical injury of pancreatic ducts and acini, (4) bacteria brought by imaging equipment or liquid infection in pancreatic duct or triggering original inflammation, (5) edema around pancreatic duct openings due to excessive coagulation in duodenal EST (EST) and impeding outflow of pancreatic secretion. Theoretically, post-ERCP pancreatitis could be reduced by mitigating papilla edema, keeping pancreatic and bile ducts open, controlling pancreatic secretion, avoiding contact of pancreatic tissue with active enzymes. Glyceryl trinitrate can relax smooth muscles not only in vascular wall but also in gastrointestinal tract, especially in the sphincter of Oddi. Sublingual glyceryl trinitrate shows its effect in 1-2 min and maintains its effect for 30 min. It also relaxes the sphincter of pancreatic and bile ducts when ERCP is performed, thus helping intubation and reducing spasm of sphincter of Oddi, keeping ducts open for contrast agent and pancreatin drainage, and reducing post-ERCP pancreatitis.

Sudhindran *et al*[10] suggested that sublingual glyceryl trinitrate (2 mg) before ERCP could relax sphincters, induce intubation and reduce 10% postoperative pancreatitis. Our study revealed that sublingual glyceryl trinitrate (5 mg) before ERCP could reduce pancreatitis and hyperamylasemia. Kaffes *et al*[11] showed that ransdermal GTN could not improve the success rate of ERCP cannulation or prevent post-ERCP pancreatitis in either average or high-risk patient groups.

There was a significant difference between the study and placebo groups. Compared with other drugs, glyceryl trinitrate is inexpensive, convenient and has less side-effects, and can be used as a prospective drug for preventing post-ERCP pancreatitis.

COMMENTS

Background

Endoscopic retrograde cholangiopancreatography (ERCP) is a widely applied method for the diagnosis and treatment of pancreatobiliary disease. Post-ERCP pancreatitis is the most common postoperative complication of ERCP and how to prevent it has become an urgent clinical challenge.

Research frontiers

ERCP is an indispensable method for the diagnosis and treatment of hepatic and pancreatobiliary disease, and pancreatitis is the most common postoperative complication of it. There are some studies on drugs for preventing post-ERCP pancreatitis, but their results remain debatable. Therefore, most endoscopy centers do not give patients a conventional preventive drug therapy.

Innovations and breakthroughs

This trail revealed that sublingual glyceryl trinitrate (5 mg) before ERCP could reduce pancreatitis and hyperamylasemia.

Applications

Sublingual glyceryl trinitrate (5 mg) 5 min before the ERCP can prevent post-ERCP pancreatitis. Compared with other drugs, glyceryl trinitrate is inexpensive, convenient and has less side-effects, and can be as a prospective drug for preventing post-ERCP pancreatitis.

Terminology

Post-ERCP pancreatitis stands for post-endoscopic retrograde cholangio-pancreatography pancreatitis; ERCP stands for endoscopic retrograde cholangi opancreatography.

Peer review

Pancreatitis is the most common postoperative complication of ERCP. This study showed that glyceryl trinitrate could relax the sphincter of pancreatic and bile ducts when ERCP was performed, thus helping intubation and reducing the spasm of sphincter of Oddi, keeping ducts open for contrast agent and pancreatin drainage, and reducing post-ERCP pancreatitis. Glyceryl trinitrate is inexpensive, convenient and has less side-effect, and can be used a prospective drug for preventing post-ERCP pancreatitis.

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Impact of *p27mt* gene on transplantation model of human colorectal cancer in nude mice

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Abstract

AIM: To investigate the inhibitory and anti-metastatic effect of mutant p27 gene (*p27mt*) on the growth of colorectal cancer xenografts in nude mice and its underlying mechanism.

METHODS: Inhibitory effect of *p27mt* gene on the growth of colorectal cancer xenografts was determined by measurement of tumor size before and after direct intra-tumoral injection of Ad-p27mt in a pre-established transplantation model of human colorectal cancer in nude mice. Cell cycle and apoptosis were detected by flow cytometry performed on single-cell suspension from an isolated tumor. Expression of MMP-9 in tumor tissue was detected by immunohistochemistry.

RESULTS: The average sizes of transplantation tumors were 1.94 ± 0.67 cm^3, 2.75 ± 0.83 cm^3 and 3.01 ± 0.76 cm^3 in the Ad-p27mt, Ad-LacZ and control groups, respectively ($P < 0.05$). The average proliferation rates were 37.34% ± 1.45%, 53.16% ± 3.27% and 54.48% ± 2.43%, in the Ad-p27mt, Ad-LacZ and control groups, respectively ($P < 0.05$). The average apoptosis rates were 19.79% ± 3.32%, 6.38% ± 4.91% and 7.25% ± 5.20% in the Ad-p27mt, Ad-LacZ and control groups, respectively ($P < 0.01$). The average MMP-9 expression rates were 20%, 75% and 66.7% in the Ad-p27mt, Ad-LacZ and control groups, respectively ($P < 0.01$).

CONCLUSION: *p27mt* inhibits the growth of transplanted tumor by blocking the proliferation of cancer xenografts and by promoting apoptosis of transplantated tumor cells, as well as decrease transplanted tumor metastasis.

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Key words: Colorectal cancer; *p27mt* gene; Nude mice; MMP-9

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Chen J, Ding WH, Lu GX, Xu SY. Impact of *p27mt* gene on transplantation model of human colorectal cancer in nude mice. *World J Gastroenterol* 2009; 15(3): 369-372 Available from: URL: http://www.wjgnet.com/1007-9327/15/369.asp DOI: http://dx.doi.org/10.3748/wjg.15.369

INTRODUCTION

Along with the improvement of people's living standard and change in diet, there has been a gradual increase in the incidence of colorectal cancer[1]. None of the current treatment modalities for colorectal cancer, including surgery, radiotherapy and chemotherapy, is effective. With the advent of post-genomic era, the function of genes has become a priority research area and brought the dawn in gene therapy for tumor. Since *p27* is an anti-oncogene, this study was to evaluate the inhibitory and anti-metastatic effect of *p27mt* gene on the growth of colorectal cancer xenografts in nude mice and its underlying mechanism and to provide the theoretical basis for the use of *p27* in clinical treatment of colorectal cancer.

MATERIALS AND METHODS

Cell line and adenovirus

Lovo cell line, purchased from the Center for Type Culture Collection of Wuhan University, was cultured in RPMI 1640 medium. Working density of Levo cells

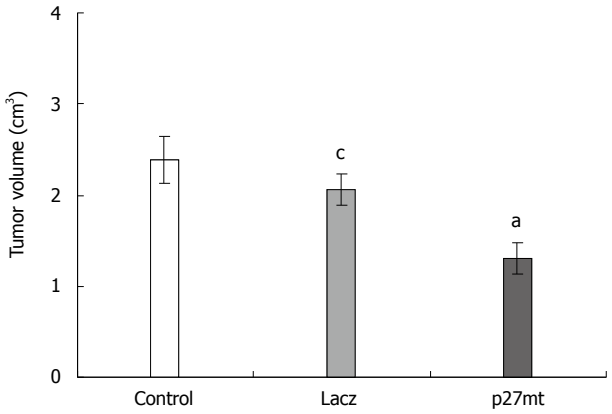

Figure 1 Comparison of transplanted tumor volume among different groups (cm^3). $^aP < 0.050$ *vs* Ad-Lacz, $^cP > 0.05$ *vs* control.

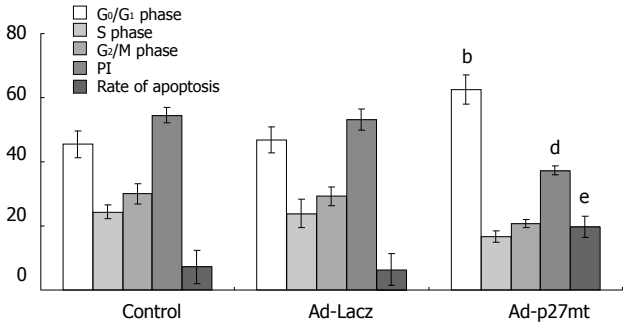

Figure 2 Comparison of status of cell cycle, PI and rate of apoptosis between different groups. $^bP < 0.01$ *vs* Ad-LacZ, $^dP < 0.01$ *vs* Ad-LacZ, $^eP < 0.01$ *vs* Ad-LacZ.

was 1×10^8/mL, with living cell count by trypan blue > 99%. Ad-LacZ was constructed and presented by Wang *et al*[2]. Ad-p27mt was self-constructed[3].

Establishment of transplantation model of colorectal cancer in nude mice and grouping

Thirty-six BALB/C nude mice, 4-6 wk old, and weighing 18-25 g, were purchased from the Laboratory Animal Management Center of Hubei Province. Lovo cell suspension (0.2 mL) was inoculated subcutaneously at the right back skin of each nude mouse. Upon tumor development, 27 nude mice whose tumor size was 0.5-1.5 cm in diameter were randomly assigned to control group, Ad-LacZ group or Ad-p27mt group. PBS (0.1 mL), Ad-LacZ (0.1 mL) with a virus density of 10^{10} pfu/mL, or Ad-p27mt (0.1 mL) with a virus density of 10^{10} pfu/mL was directly injected into the tumor of nude mice in the three groups, respectively, once every 3 d, for 28 d.

Determination of transplanted tumor size

Transplanted tumor size was calculated according to the following formula: $V = ab^2/2$, where a and b represent the length and width of the xenograft, respectively.

Flow cytometry

The 28th day after virus injection into the transplanted tumor, all mice were sacrificed with their tumors removed, weighed and photographed. Tumor tissue (15 g) was used in preparation of single cell suspension. Two hundred μL DNA-PREP™ LPR was mixed with 100 μL single cell suspension, and 1000 μL DNA-PREP stain was added into the mixture 3 min after the mixture was set at room temperature and protected from light. Fifteen min later, cell cycle and apoptosis were determined with a Coulter Epics XL flow cytometer. Proliferation index (PI) was calculated according to the following formula[4]:

$$PI = (S + G_2/M)/(G_0/G_1 + S + G_2/M) \times 100\%$$

Immunohistochemical detection of MMP-9 expression

Anti-human mouse MMP-9 monoclonal antibody, S-P staining kit and DAB developer were obtained from Beijing Zhongshan Biotechnology, Co, Ltd. Since MMP-9 appears to be brown granules in cytoplasm, total cell number and the number of MMP-9 positive cells were counted in 5 visual fields of the matrix area around the tumor nest under microscope. Based on the scope and extent of staining, immunohistochemical results were logged according to the following criteria: "-" - no positively stained cells; "+" - cells lightly stained or < 10% cells stained; "++" - moderately stained or 10%-25% cells stained; "+++" - darkly stained or more than 50% cells stained, where - represents negative expression, +/++ stands for weakly positive expression, and + + + stands for strong expression.

Statistical analysis

One way-ANOVA was used in processing measurement data, which were expressed as mean ± SD. χ^2 test was adopted in calculation of enumeration data.

RESULTS

Comparison of transplanted tumor size between different groups

The average size of transplanted tumor in the Ad-p27mt group (1.94 ± 0.67 cm^3) was significantly smaller than that in the control group (3.01 ± 0.76 cm^3) ($P < 0.05$), no statistical significance was found in the average size of transplanted tumor between the two groups (3.01 ± 0.76 cm^3 *vs* 2.75 ± 0.83 cm^3) (Figure 1).

Comparison of cell cycle, PI and apoptosis between different groups

More cells at G_0/G_1 phase and less cells at S and G_2/M phase were observed in the Ad-p27mt group than the other two groups ($P < 0.05$). However, the difference between the two groups was insignificant ($P < 0.05$, Figure 2).

MMP-9 expression in transplanted tumor

Since MMP-9 is mainly found in the matrix area around tumor nest, brown stained granules were observed in cytoplasm of cancer cells (Figure 3). MMP-9 expression rate for the Ad-p27mt group was significantly decreased compared with control group (Table 1).

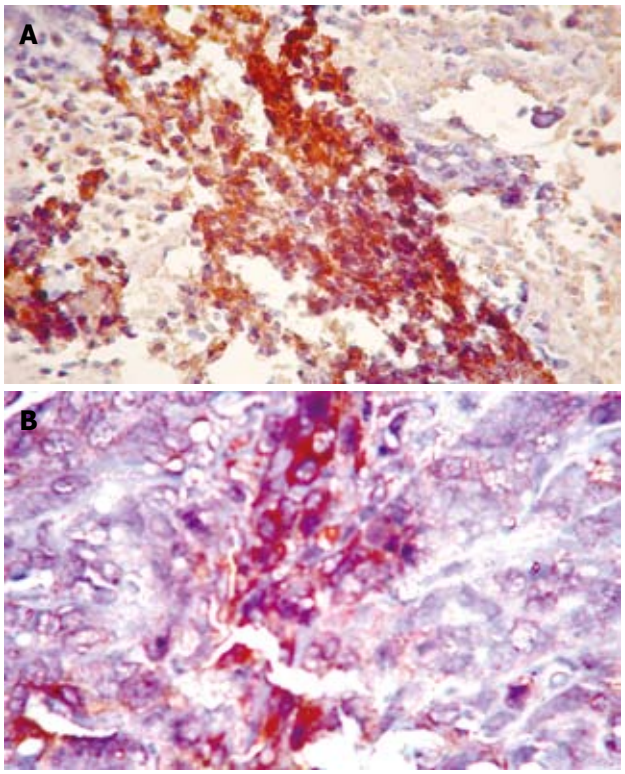

Figure 3 Expression of MMP-9 in the transplanted tumor. A: control group; B: Ad-p27mt group.

DISCUSSION

While *p27* is a negative regulator of cell cycle and a tumor suppressor[5], tumor may develop when abnormal (missing or decreased) expression of *p27* and attenuated inhibition on cell cycle lead to uncontrolled cell growth and carcinogenesis[6]. The results of studies demonstrate that decreased *p27* expression was associated with ubiquitin-mediated proteasome phosphorylation and abnormal activity of *p27*[7,8]. We investigated the *in vivo* inhibitory effect of *p27* on transplanted tumor by intratumoral injection of mutated *p27mt* adenovirus.

Park *et al*[9] found that inhibition of mutant *p27* (*p27mt*) on tumor cells seem stronger than that of wild type *p27* (*p27wt*) as demonstrated in cells arrested in G_0/G_1 phase, and that the apoptosis promoting activity of *p27mt* is also stronger. Another study revealed that the half-life of *p27mt* is over 12 h, much longer than that of *p27wt* (2 h)[10]. Through determination of the size of transplanted tumor, this study displayed that *p27mt* gene significantly inhibited the growth of colorectal cancer by inhibiting cell proliferation and by promoting cell apoptosis, suggesting that *p27mt* can evidently suppress cell proliferation at G_0/G_1 phase. The apoptosis promoting activity of *p27mt* was more obvious in control group, while the apoptosis rate of *p27wt* was up to 37.9% ± 3.32%.

It was reported in our preliminary study that the expression level of *p27* in colorectal cancer tissue is quite low[11]. In this study, the expression of MMP-9 was significantly decreased in *p27mt* group. MMP-9, in the form of proenzyme in cytoplasm, when released under physiological condition, may degrade extracellular matrix and is involved in development of human body and multiple physiological processes including tissue repair[12]. When disturbance of MMP-9 gene occurs, increased proenzyme leads to escalated degradation of extracellular components, including Ⅳ and Ⅴ collagen and laminin, and undermined integrity of basement membrane. Therefore, MMP-9 plays a very important role in the process of tumor metastasis[13]. In this study, a reduced MMP-9 expression was observed after *p27mt* was injected. No tumor metastasis was found with in 28 d after transplantation of the tumor.

In conclusion, *p27mt* inhibits the growth of colorectal cancer by inhibiting cancer cell proliferation and promoting cell apoptosis as well as metastasis of colorectal cancer.

Table 1 Comparison of MMP-9 positive rate between different groups

Group	-	+	++	+++	Positive rate (%)
Control	12	4	11	9	66.7
Ad-LacZ	9	2	14	11	75[a]
Ad-p27mt	30	2	4	0	20[c]

[a]$P > 0.05$ *vs* control group; [c]$P < 0.05$ *vs* Ad-LacZ group and control group.

COMMENTS

Background

Along with the improvement in people's living standard and changes in diet, there has been a gradual increase in the incidence of colorectal cancer in China. However, no effective therapeutic modalities are available for it. Gene therapy for restoration of *p27* expression is a promising therapy for it. A mutant type of p27 gene, with mutant of Thr-187/Pro-188 to Met-187/Ile-188, can inhibit degradation of *p27* protein through the ubiquitin-mediated pathway. The inhibitory effect of mutant *p27 (p27mt)* seems stronger than that of wild type *p27 (p27wt)* on tumor cells, as demonstrated by cells arrested in the G_0/G_1 phase. The apoptosis promoting activity of *p27mt* is also stronger. However, no study about its effect on colorectal cancer is available.

Research frontiers

p27, a cyclin-dependent kinases inhibitor, a tumor suppressor gene, and a promoter of apoptosis, has been widely investigated. Anti-tumor activity of *p27* has been demonstrated in breast, lung, and oral cancer. However, the anti-tumor bioactivity of *p27mt* has not been studied on colorectal cancer.

Innovations and breakthroughs

The results of this study indicate that *p27mt* gene has a strong anti-tumor bioactivity on colorectal cancer *in vivo* and *in vitro*.

Applications

This gene may be developed into a new therapeutic agent for colorectal cancer.

Peer review

This study showed the effect of over-expression of a mutant form of *p27* on colorectal cancer growth in a xenotransplantation model. The results are largely descriptive and the effect of *p27mt* seems modest on tumor growth. Further study is needed to show the expression of transfected *p27mt* gene.

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CASE REPORT

Pedunculated Brunner's gland hamartoma of the duodenum causing upper gastrointestinal hemorrhage

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Abstract

A case of pedunculated Brunner's gland hamartoma (BGH) of the duodenum causing upper gastrointestinal (GI) hemorrhage is reported. The patient was a 47-year-old man who visited our hospital for further evaluation of tarry stools and shortness of breath. Endoscopic examination of the upper digestive tract revealed a large peduncular polyp with bleeding, about 30 mm in diameter, arising from the wall of the second portion of the duodenum. GI bleeding occurred from the base of the stalk of the polyp. Endoscopic polypectomy was performed. Histological examination of the specimen revealed that the main body of the polyp contained several lobules of mature Brunner's gland with areas of cystic dilatation. The surface epithelium consisted of normal duodenal mucosa with areas of focal ulceration. This polyp was diagnosed as a BGH. The symptom of tarry stools resolved after endoscopic resection. Our case shows that treatment is necessary for duodenal BGH if GI bleeding occurs.

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Key words: Brunner's gland; Hyperplasia; Duodenal polyp; Endoscopic polypectomy; Gastrointestinal bleeding

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Hirasaki S, Kubo M, Inoue A, Miyake Y, Oshiro H. Pedunculated Brunner's gland hamartoma of the duodenum causing upper gastrointestinal hemorrhage. *World J Gastroenterol* 2009; 15(3): 373-375 Available from: URL: http://www.wjgnet.com/1007-9327/15/373.asp DOI: http://dx.doi.org/10.3748/wjg.15.373

INTRODUCTION

Brunner's gland hyperplasia and hamartoma are infrequently encountered polypoid nodules and masses in the proximal duodenum[1]. Brunner's gland hamartoma (BGH) is a very rare cause of upper gastrointestinal (GI) hemorrhage. Clinically, patients may present with symptoms of duodenal obstruction or upper GI hemorrhage and require endoscopic or surgical excision[2]. Herein, we describe a relatively rare case of pedunculated BGH of the duodenum causing upper GI hemorrhage.

CASE REPORT

A 47-year-old man presented with the symptoms of tarry stools and shortness of breath. He was in good health with no specific family or past medical history. His body temperature was 36.5℃, blood pressure was 148/82 mmHg, and radial pulse rate was 70 beats/min and regular. He had anemia, but no jaundice. Neurological examination revealed no abnormal findings. Laboratory tests showed a red blood cell count of $318 \times 10^4/\mu L$ [normal range (NR), 430×10^4-$570 \times 10^4/\mu L$], a white blood cell count of $7600/\mu L$, a platelet count of $30.8 \times 10^4/\mu L$, and a hemoglobin concentration of 8.9 g/dL (NR, 14-18 g/dL). Endoscopic examination of the upper digestive tract revealed a large peduncular polyp, about 30 mm in diameter, arising from the wall of the second portion of the duodenum (Figure 1A). The polyp head was lobulated. GI bleeding occurred from the base of stalk of the polyp (Figure 1B and C). It was suspected to be a BGH, from the endoscopic findings. An air-contrast barium meal also revealed a pedunculated polyp in the second portion of the duodenum (Figure 2). There was no lesion in the esophagus and stomach. Endoscopic polypectomy was performed (Figure 3). The cut surface of the resected specimen showed an approximately 3-cm whitish mass (Figure 4). Histological examination of the specimen revealed that the main body of the polyp contained several lobules of mature Brunner's gland with areas of cystic dilatation (Figure 5). The surface

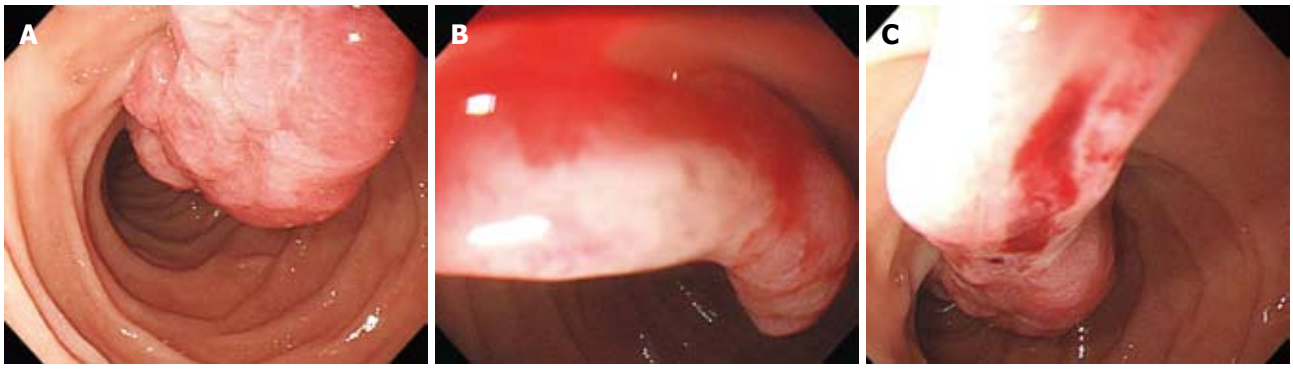

Figure 1 Endoscopy images. A large lobulated peduncular polyp, about 30 mm in diameter, in the second portion of the duodenum (A and B) and bleeding from the base of the stalk of the polyp (C).

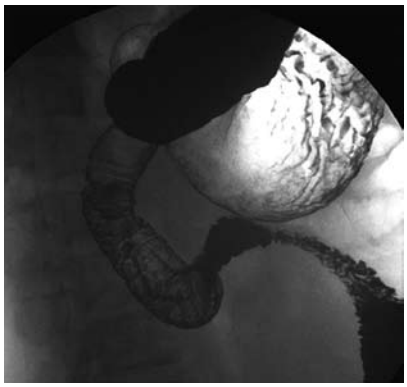

Figure 2 Double contrast radiograph of the duodenum showing an approximately 3-cm peduncular polyp.

Figure 3 Endoscopic polypectomy was performed and the resected specimen was moved into the stomach.

Figure 4 Macroscopic findings of the polypectomy specimen. The cut surface showed an approximately 3-cm whitish mass.

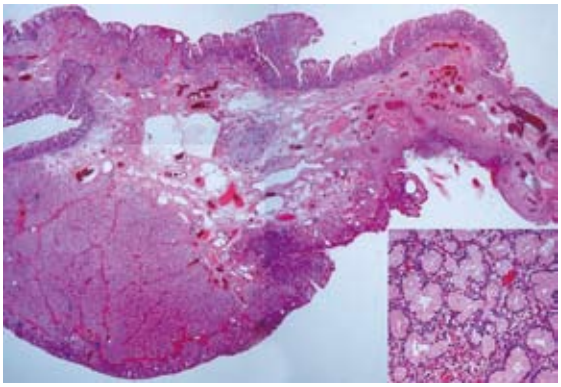

Figure 5 Microscopic findings of the polypectomy specimen. Low-power view of a cross section showing submucosal proliferation of Brunner's gland below the duodenal mucosa (HE x 10, x 100).

epithelium consisted of normal duodenal mucosa with areas of focal ulceration. The base of the stalk was ulcerated. There was no evidence of malignancy. The lesion was diagnosed as a pedunculated BGH of the duodenum. After endoscopic polypectomy, the symptom of tarry stools resolved.

DISCUSSION

The etiology of BGH remains obscure. BGH is present mostly in middle age without any gender predominance[3]; however, cases have been described from early infancy to even 80 years of age. BGHs have a broad range of sizes (0.5-12 cm)[1,4-6]. The most common location is the posterior wall of the duodenum near the junction of the first and second portions. BGHs were found in the duodenal bulb in 70% of cases, in the second portion of the duodenum in 26%, and in the third portion in 4% in one series of 27 patients by Levine *et al*[3]. In the present case, it was located in the second portion of the duodenum.

BGHs are usually asymptomatic and often detected

incidentally on barium meal or endoscopy[1,6]. Another review of the literature has revealed that the main clinical feature of BGH is GI bleeding or bowel obstruction[1,2,4,6,7]. The diagnosis of BGH is usually made by a combination of radiographic and endoscopic findings. The radiographic finding of large localized BGHs is a sessile or pedunculated polypoid filling defect[1,6,8]. Endoscopic characteristic findings, like radiographic findings, are as follows: (1) pedunculated polyp, although 11% can be sessile[3,9,10]; (2) polypoid or lobulated mass; and (3) covered with normal mucosa[3,11,12]. BGH should be distinguished from other duodenal lesions such as leiomyoma, polypoid adenoma of the superficial mucosal glands, aberrant pancreatic tissue and malignant tumors[1,4,13,14]. Diagnosis is rarely conclusive on endoscopic biopsies because the lesion locates mainly in the submucosal layer, and the biopsy is often not deep enough to reach the submucosal tumor tissue[1,6,13]. The final diagnosis of BGH depends on the pathological findings of resected specimens obtained by endoscopic mucosal resection, polypectomy or surgical treatment.

As to therapy, BGH of the duodenum can best be removed endoscopically, because it is thought to be clinically and histologically benign. However, endoscopists should be aware that there have been rare case reports of malignancy arising from Brunner's gland[1,4,15,16]. Endoscopic or surgical treatment is necessary if GI bleeding occurs[4,7,11-14], as in the present case. GI bleeding, typically manifested by hematemesis or melena, from ulceration or erosion of the mucosa stretched over the submucosal lesion, may occur and can occasionally be massive and rarely fatal[3,10,13]. In the present case, bleeding was caused by ulceration of the stalk. The mechanism underlying GI bleeding in the present case was atypical.

In conclusion, we report a case of pedunculated BGH of the duodenum causing upper GI hemorrhage. BGH should be generally taken into consideration as a differential diagnosis of duodenal masses. BGH is not fatal and patients remain asymptomatic in their daily lives, except for GI bleeding or bowel obstruction. Endoscopists should be aware that BGH may exhibit the aforementioned endoscopic characteristics and may cause GI bleeding.

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LETTERS TO THE EDITOR

Agenesis of the dorsal pancreas

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Abstract

During the last 100 years in medical literature, there are only 54 reports, including the report of Pasaoglu *et al* (*World J Gastroenterol* 2008; 14: 2915-2916), with clinical descriptions of agenesis of the dorsal pancreas in humans. Agenesis of the dorsal pancreas, a rare congenital pancreatic malformation, is associated with some other medical conditions such as hyperglycemia, abdominal pain, pancreatitis and a few other diseases. In approximately 50% of reported patients with this congenital malformation, hyperglycemia was demonstrated. Evaluation of hyperglycemia and diabetes mellitus in all patients with agenesis of the dorsal pancreas including description of fasting blood glucose, oral glucose tolerance test, glycated hemoglobin and medical treatment would be a future goal. Since autosomal dominant transmission has been suggested in single families, more family studies including imaging technologies with demonstration of the pancreatic duct system are needed for evaluation of this disease. With this letter to the editor, we aim to increase available information for the better understanding of this rare disease.

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Key words: Agenesis of dorsal pancreas; Diabetes mellitus; Glucose intolerance; Abdominal pain; Pancreatitis

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TO THE EDITOR

We read with interest the published case report of Pasaoglu *et al* on agenesis of the dorsal pancreas[1]. Agenesis of the dorsal pancreas is a rare congenital pancreatic malformation and may be associated with some other medical conditions and diseases. During the last 100 years in medical literature, we know of 54 reports with clinical descriptions of agenesis of the dorsal pancreas in humans. We recently summarized systematically all reported patients with agenesis of the dorsal pancreas and discussed the associated medical conditions and diseases[2]. In 1911, the first description of agenesis of the dorsal pancreas was published as an autopsy finding. Now, as new imaging technologies have been developed and improved, the number of patients reported to show agenesis of the dorsal pancreas has increased rapidly over the last years. So far, the findings in autopsy and the radiological descriptions of anatomic pancreatic structures are highly variable and in most cases there is a diagnosis made without description of the pancreatic duct system[2]. In some patients, an enlarged or prominent or compensatory hypertrophy of the pancreatic head is described, whereas other descriptions include normal sized pancreatic head, as well as mildly atrophic and small head of the pancreas. We support the description of Pasaoglu *et al*[1] and confirm that the diagnosis of agenesis of the dorsal pancreas is inconclusive without demonstration of the absence of the dorsal pancreatic duct, either with endoscopic retrograde or magnetic resonance pancreatography.

Diabetes mellitus comprises a group of metabolic diseases characterized by hyperglycemia resulting from

defects in insulin secretion and/or insulin action. Criterion for diagnosis of diabetes mellitus is a fasting blood glucose > 126 mg/dL. Another diagnostic test to differentiate impaired glucose tolerance and diabetes mellitus is the oral glucose tolerance test[3]. Single familial observations of agenesis of the dorsal pancreas suggest autosomal dominant transmission. In one family with agenesis of the dorsal pancreas, a marked defect in hepatic glycogen metabolism, even in non-diabetic offspring, is demonstrated. An impaired index of the first phase insulin secretion in the diabetic and in both non-diabetic family members is described. Since the root cause of most common diabetes mellitus, type 1 and type 2 diabetes, is a decrease in-cell mass, this can be related to reduced-cell mass and might contribute to the development of glucose intolerance which ultimately leads to diabetes mellitus[4].

We suggest that more family studies including imaging technologies with demonstration of the pancreatic duct system are needed. Evaluation of hyperglycemia in all patients with agenesis of the dorsal pancreas including description of fasting blood glucose, oral glucose tolerance test, glycated hemoglobin and medical treatment would be a future goal.

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LETTERS TO THE EDITOR

Another new variant of Bouveret's syndrome

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Abstract

Although Bouveret's syndrome, i.e. gastric outlet obstruction by a large gallstone impacted in the proximal duodenum secondary to a cholecystoduodenal fistula, is rare, its pathogenesis and clinical features are well characterized. However, existence of variant forms of the syndrome are not well known, and as far as we know, only two cases of variant Bouveret's syndrome have been described in the English-language literature. We present a case of another new variant of Bouveret's syndrome in a 54-year-old Korean woman.

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Key words: Duodenal obstruction; Biliary fistula; Gallstones

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TO THE EDITOR

We read with much interest the case report of Arioli *et al*[1], 'a new variant of Bouveret's syndrome', recently published in *World Journal of Gastroenterology*. They described a case of gallstone ileus in which an incomplete pyloric occlusion developed following the migration of a large stone through a cholecystogastric fistula, in a patient with undiagnosed chronic cholecystitis. In their case, the gallstone lay in the gastric antrum rather than within the lumen of the duodenum, which is different from typical Bouveret's syndrome. Doody *et al*[2] have also described a variant of Bouveret's syndrome in which duodenal obstruction was caused by a huge gallstone that lay outside the duodenum, which gave an atypical Bouveret's syndrome appearance. We recently encountered a similarly interesting case of gastric outlet obstruction caused by complicated gallstone disease. A 54-year-old Korean woman was admitted to the hospital with epigastric pain and intermittent vomiting of 20 d duration. She had a history of recurrent bouts of right

Figure 1 Gastroduodenal endoscopy revealed an ovoid mass in the first portion of the duodenum, the center of which harbored a mucosal defect.

Figure 2 Abdominal CT showed wall thickening at the bulbar and postbulbar portions of the duodenum, with marked gastric dilatation. A cystic lesion (arrow) was found at the thickened duodenal wall. The gallbladder was partially collapsed and adhered to the duodenum.

upper quadrant abdominal pain caused by acute cholecystitis. Gastroduodenoscopy showed an obstructive duodenal mass, the center of which harbored a mucosal defect (Figure 1). Abdominal computed tomography (CT) showed a cystic lesion within the thickened wall of the bulbar and post-bulbar portions of the duodenum (Figure 2). The gallbladder contained a small stone and was partially collapsed. On laparotomy, the gallbladder was densely adhered to the first portion of the duodenum. The patient underwent cholecystectomy, with excision of a portion of the duodenal wall. In the cystic lesion of the duodenal wall, muddy stone fragments were found. Based on operative findings, it was certain that a large gallstone had passed through the cholecystoduodenal fistula into the duodenal wall, and remained there sufficiently long to form a submucosal mass that eroded through the mucosa into the duodenal lumen. Although the stone was passed without causing any further problems, the cavaitary lesion in the duodenal wall persisted and bulged into the duodenal lumen, which caused the duodenal obstruction. The features of our patient resembled a Bouveret's syndrome in clinical presentation, but differed from it inasmuch that the duodenal obstruction was not caused by a gallstone per se, but rather by secondary changes in the duodenal wall caused by a gallstone. A cystic lesion within the wall of the duodenum caused confusion in our case, and surgical exploration was required to make a definite diagnosis and to determine a treatment strategy. We think that this case may be another new variant of Bouveret's syndrome.

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Meetings

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Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

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

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

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

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

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

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

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

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

March 20-24, 2009
Marriott Wardman Park Hotel
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13th International Symposium on Viral Hepatitis and Liver Disease

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Email: bsg@mailbox.ulcc.ac.uk

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

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

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

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

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

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

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

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

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

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

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

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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

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

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EDITORIAL

Intestinal hormones and growth factors: Effects on the small intestine

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Abstract

There are various hormones and growth factors which may modify the intestinal absorption of nutrients, and which might thereby be useful in a therapeutic setting, such as in persons with short bowel syndrome. In part Ⅰ, we focus first on insulin-like growth factors, epidermal and transferring growth factors, thyroid hormones and glucocorticosteroids. Part Ⅱ will detail the effects of glucagon-like peptide (GLP)-2 on intestinal absorption and adaptation, and the potential for an additive effect of GLP2 plus steroids.

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Key words: Epidermal growth factor; Glucocorticosteroids; Insulin-like growth factor-Ⅰ/Ⅱ; Intestinal growth; Transforming growth factor-α-2; Hepatocyte growth factor; Keratinocyte growth factor

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INTRODUCTION

In this review, the hormones and growth factors to be reviewed which alter nutrient absorption include insulin-like growth factor Ⅰ (IGF-Ⅰ) and Ⅱ, EGF, TGF-α, HGF, Erythropoietin and KGF. In Table 1 is outlined the cell types which express those peptides, as well as the cell types in the intestine and elsewhere which express the corresponding receptors. Part Ⅰ there are various hormones and growth factors which may modify the intestinal absorption of nutrients, and which might thereby be useful in a therapeutic setting, such as in persons with short bowel syndrome. Part Ⅱ presented here will detail the effects of glucagon-like peptide (GLP)-2 on intestinal absorption and adaptation, and the potential for an additive effect of GLP2 plus steroids.

INSULIN-LIKE GROWTH FACTORS (IGF-Ⅰ, IGF-Ⅱ)

The insulin-like growth factor family consists of insulin, IGF-Ⅰ, and IGF-Ⅱ. The liver is the major site of synthesis for IGF-Ⅰ, but it is also synthesized in other tissues including the gastrointestinal tract[1,2]. IGF-Ⅰ and IGF-Ⅱ are found in human milk[3], and these peptides are members of a complex mixture of growth factors that the neonate is exposed to during the suckling period.

Intestinal development may involve IGF-Ⅰ and IGF-Ⅱ[4-6]. IGF-Ⅰ receptor (IGF-1R) is detectable in the fetus and the neonate of animals[7,8]. In humans, IGF-Ⅰ, IGF-Ⅱ and IGF-IR are also present in fetal intestine[9-11].

Intestinal cells proliferate *in vitro* when exposed to IGF-Ⅰ[12-14]. Transgenic mice that over express IGF-Ⅰ demonstrate increased small intestinal growth[15], particularly in the muscle layers of the distal small intestine and large intestine[16]. IGF-Ⅰ receptor gene knockout animals have fetal growth retardation, but distinctive effects on the gastrointestinal system have not been found[17]. This may indicate the presence of redundant systems in the gastrointestinal tract that compensate for the lack of IGF-Ⅰ.

In vivo studies show variable responses depending on the dose of IGF-Ⅰ that is used. Pharmacological doses of IGF-Ⅰ, given orally to colostrum-deprived 5 d old piglets, increase electrolyte and nutrient absorption[18]. Neonatal pigs fed formula supplemented with IGF-Ⅰ from birth to 4 d of age had increased mucosal growth[19]. Parenterally fed 1 d old piglets given enteral IGF-Ⅰ had increased brush border malease (BBM) disaccharidase levels[20]. Houle *et al*[21] administered IGF-Ⅰ orally to neonatal rats, and demonstrated increases in mucosal growth and BBM enzymes. Similarly, Burrin *et al*[19] showed increases in intestinal weight, protein, and DNA in neonatal pigs fed

Table 1 Hormones and peptides which affect intestinal nutrient absorption

Hormones/ Peptides	Cell types which express the specific peptide	Cell types in the intestine and elsewhere which express corresponding receptors
IGF Ⅰ & Ⅱ	Granulosa cells[158] Prostate stromal cells[159] Neonatal bone marrow-mesenchymal stem cells[160] Systemic sclerosis lung fibroblasts[161] Melanoma Cells (SK-MEL2)[162]	Non-Islet cell tumors[163] Hepatoma cells[164] Intestinal smooth muscle[165] Astrocytoma cells[166] Camel intestinal lamina propria[167] Camel epithelia of the crypt cells[167] Camel villi[167]
EGF	Rat submandibular glands cells[168] HaCaT cells[169] Porcine cumulus cells[170] Esophageal epithelial cell[171]	Keratinocytes[169] Urothelial cells[172] Fibromyoblasts[173] Smooth muscle cells[173] Fibroblasts[173] Colonic epithelial cells[174] Murine pancreatic beta cells[175]
TGF-α	Hypothalamic astrocytes[176] Granulosa cells[177] Ovarian theca cells[177] Endometrial stromal cells[177] Eosinophhils[178] Colon cancer cells[179]	Pancreatic cancer cells[180] Osteoblasts[181] Murine conjunctival goblet cells[182] Fundic mucous cells[183] Murine intestinal epithelial cells[184]
HGF	Lung microvascular endothelial cells[185] Umbilical vein endothelial cells[185] Neutrophils[186] Monocytes[187] Adipocytes[188]	Cardiomyocytes[189] Liver progenitor cells[190] Glioblastoma cells[191] Myeloma cells[192] Tumor plasma cells[192] Neuroblasts[193] Hepatocellular carcinoma cells[194] Epithelial tubular cells[195] Antroduodenal G cells[196] Rectal enterochromaffin cells[196] Pancreatic A and B cells[196] Intestinal epithelial cells[196]
Erythropoietin	Amniotic epithelial cells[197] Schwann cells[198] Interstitial fibroblasts[199] Metanephric adenoma cells[200] Mesenchymal stromal cells[201]	Erythroid cells[202] Endothelial cells[203] Neuronal cells[203] Cardiac myocytes[203] Vascular smooth muscles[203] Murine fetal enterocytes[204] Intestinal epithelial cells[205]
KGF	Mucosal and dermal fibroblasts[206] Gastric fibroblasts[207] Endometrial stromal cells[208]	Alveolar epithelial type II cells[209] Breast cancer cells[210] Cholesteatoma cells[211] Enterochromaffin cells[212]

Note: All of these are in humans until stated otherwise.

orogastrically. Alexander and Carey[18] examined the effect of oral IGF- I on neonatal piglet intestine, and found increases in D-glucose uptake in everted jejunal sleeves, independent of the intestinal mass or surface area. Lane *et al*[22] used RT-PCR and laser scanning confocal microscopy to show that intragastric IGF- I or IGF- Ⅱ increased the expression of the glucose transporters SGLT1 and GLUT2 in suckling rat intestine.

The mechanisms responsible for increased jejunal transport rates observed in tissues treated with orally administered IGF- I may include the increases in Na^+K^+-ATPase seen with IGF- I treatment. The PI3-kinase pathway may also be important, as preincubation with a PI3-kinase inhibitor abolishes the effects of IGF- I on ion and nutrient transport[23].

Although pharmacological doses of IGF- I have clear effects on the neonatal intestine, studies using physiological doses show only limited effects. For example, recent studies with both neonatal pigs[21,24] and calves[25] have shown that the oral administration of physiological concentrations of IGF- I in formula results in measurable increases in crypt cell proliferation, but there are no demonstrable increases in intestinal mucosal mass or length.

EPIDEMAL GROWTH FACTOR AND TRANSFORMING AND TRANSFORMING GROWTH FACTOR-α (TGF-α)

EGF and TGF-α are two ligands of the EGF receptor

that have been postulated to play a role in intestinal ontogeny. Both peptides are present in human breast milk, with TGF-α at a 5-10-fold lower concentration than EGF[26]. Transforming growth factor β (TGFβ) is a family of growth factors, with the three isoforms (TGFβ1, TGFβ2, TGFβ3) having nearly identical biological activities. TGFβ1 and TGFβ2 are present in human milk[27], and mRNA and protein corresponding to the three TGFβ isoforms are found in the intestinal mucosa[28-30]. Small intestinal development, however, is not severely impaired by the targeted disruption of the TGFβ gene[31].

EGF and TGF-α have been isolated in human 15-20 wk gestation fetal intestine, with TGF-α levels as much as 10 times higher than EGF[32]. EGF receptor expression is detectable throughout the GI tract in the human fetus and neonate. EGF receptor is developmentally regulated with expression being low during the human suckling period, and in rodents, unlike humans, EGF receptor expression is delayed until weaning[33].

Oral EGF administration augments gut growth and functional development in neonatal rats and pigs[34-36]. Gene targeting studies have demonstrated that disrupting the expression of the EGF receptor in mice resulted in impaired intestinal development, and death associated with a necrotizing enterocolitis-like disorder[37].

The postnatal development of intestinal transport and the physical composition of the BBM were examined in New Zealand White rabbits receiving EGF (40 mg/kg per day) either intraperitoneally or orogastrically from day 3 to day 17 of life. Intestinal water, Na^+ and glucose absorption expressed per cm of intestine were significantly increased in animals receiving EGF by either route. Increased absorption induced by orogastric EGF appeared to be secondary to mucosal hyperplasia. The BBM isolated from EGF-treated animals was significantly more fluid than that of controls. These results suggest that EGF modulates the development of transport function during the postnatal period, both by stimulating mucosal growth and by inducing specific transport processes[38].

Experimental necrotizing enterocolitis (NEC) was induced by exposure of newborn animals to asphyxia and cold stress. The newborn rats were artificially fed either with growth factor-free rat milk substitute or the same formula supplemented with 500 ng/mL of EGF[39]. EGF supplementation of formula reduced the incidence and severity of NEC, as assessed by gross and histological scoring of the ileum. This finding suggests a potential therapeutic approach for the prevention and treatment of NEC.

HEPATOCYTE GROWTH FACTOR, ERYTHROPOIETIN, KERATINOCYTE GROWTH FACTOR AND THYROID HORMONES

Erythropoietin (Epo) is present in breast milk[40], and receptors for Epo are present on intestinal cells[41]. Modest changes in small intestinal length and surface area are observed following subcutaneous Epo injections in artificially fed rat pups. Hepatocyte growth factor (HGF) is also present in breast milk, and both HGF and its receptor are present in fetal intestinal tissue[42,43]. Keratinocyte growth factor (KGF) promotes proliferation and up-regulates SI expression in fetal human small intestine explant culture[44]. It remains to be seen if KGF plays a physiological role in postnatal intestinal development.

The effect of thyroid hormones on the ontogeny of BBM enzymes has been examined. An *in vivo* study of lactase phlorizin hydrolase (LPH) catalytic activity, synthesis, and degradation was performed in propylthiouracil-induced hypothyroid rat pups, hypothyroid pups injected with thyroxine, and normally weaned rats. T4 regulates LPH ontogeny by posttranslational mechanisms that include altered processing and increased degradation of the BBM lactase enzyme[45]. Monteiro *et al*[46] examined the role of T4 on the precocious enhancement of GLUT5 in weanling rats. Rat pups were made hypothyroid by giving the dam 0.01% propylthiouracil in the drinking water from day 18 of gestation. The hypothyroid pups and age-matched euthyroid control pups were then fed high-fructose solutions by gavage, twice a day starting at 17 d of age for 3 d. Although serum T4 levels were five times lower in the hypothyroid pups, the mRNA level for the BBM fructose transporter (GLUT5) increased in euthyroid and hypothyroid pups fed high fructose. This result paralleled the increase in fructose uptake. This suggests that during weaning, dietary fructose can precociously enhance intestinal fructose uptake and GLUT5 mRNA expression, independent of developmental increases in serum T4 levels.

GLUCOCORTICOSTEROIDS

Biochemistry

Steroids can be divided into two categories: glucocorticosteroids (GC), if potency is based on liver glycogen deposition, and mineralocorticoids, if potency is based on sodium retention. The hormones that are secreted in significant amounts are cortisol, corticosterone, and aldosterone. In humans, the major naturally occurring steroid is cortisol, while in rodents the main steroid is corticosterone.

Steroids are synthesized from cholesterol in the inner mitochondrial membrane of the adrenal glands, through a series of reactions including hydroxylations. The release of cortisol from the adrenal glands is regulated by the hypothalamic-pituitary-adrenal axis, and involves the secretion of corticotropin-releasing hormone from the hypothalamus, and the subsequent secretion of adrenocorticotropin hormone (ACTH) from the pituitary gland. The rate-limiting step in steroid synthesis is the transport of cholesterol to the inner mitochondrial membrane by sterol carrier protein 2 (SCP2), steroidogenesis activator protein polypeptide (SAP), peripheral benzodiazepine receptor (PBR), and

steroidogenic acute regulatory protein (StAR)[47]. Because corticosteroids are not stored in adrenal tissues, the rate of secretion equals the rate of biosynthesis[48-51].

GC are classified as lipids because they are more soluble in organic solvents than aqueous solvents. Their solubility is affected by the presence of hydroxyl or carbonyl groups[52]. GC enter cells by diffusion across the plasma membrane. About 90% of circulating cortisol is reversibly bound to a plasma protein, the corticosteroid binding globulin (CBG)[48,51,52]. CBG is a high affinity, low capacity plasma protein, while albumin has a low affinity and high capacity to bind steroids[48,51-53]. The bioavailablility of GC in specific tissues is determined by the presence of tissue specific metabolizing enzymes (11-β-hydroxysteroid dehydrogenase (11β-HSD)), CBG levels, the presence of efflux proteins (such as multi-drug resistance protein 1, MDR1), and the expression of the glucocorticosteroid receptors (GR). Other levels of regulation include variations in the receptor protein (isoforms, polymorphisms), alternative receptor dimerization, co-chaperones, levels of hsp, and posttranslational modifications[54].

Approximately 70% of corticosteroid metabolism occurs in the liver, and involves the cytochrome P450 system[55]. Intestinal metabolism is also important, as the intestinal sites of cortisol and cortisone metabolism in humans are saturated before the hepatic sites[56]. The majority of corticosteroids are excreted in the urine, although small amounts are detectable in fecal, biliary and pulmonary CO_2 excretions[48,49,57].

Glucocorticosteroid receptors (GR) are 94 kDa proteins found in the cytoplasm of cells from many tissues. When ligand binding occurs, an inhibitory protein is released, the receptor dimerizes, hyperphosphorylation occurs[58-60], and a DNA binding site is exposed (Figure 1). This conformational change, which may unmask nuclear localization signals, allows the GR to translocate to the nucleus. The GR may affect gene transcription and subsequent protein synthesis by interacting with specific nuclear binding sites (referred to as glucocorticosteroid response elements, GRE). This causes either stimulation or, less frequently, inhibition of transcription. Also, the binding of the GR homodimer to the GRE may induce rearrangement of the chromatin, allowing other transcription factors to bind to previously inaccessible DNA[61].

The GR may also interact with other transcription factors, such as AP-1, in which case transcription is commonly inhibited[62,63]. There are several other transcription factors that have been linked to the GR, including the p65 subunit of NFκB[64,65].

The unliganded GR may be associated with heat shock proteins (hsp), including hsp56, hsp70 and hsp90. There may be constant cycles of dissociation/reassociation of these components, as well as constant bi-directional shuttling of GR between the cytosol and the nucleus[66,67]. Furthermore, the action of hsp70 and hsp90 may be further regulated, either positively or negatively, by co-chaperones[68-71]. These proteins may

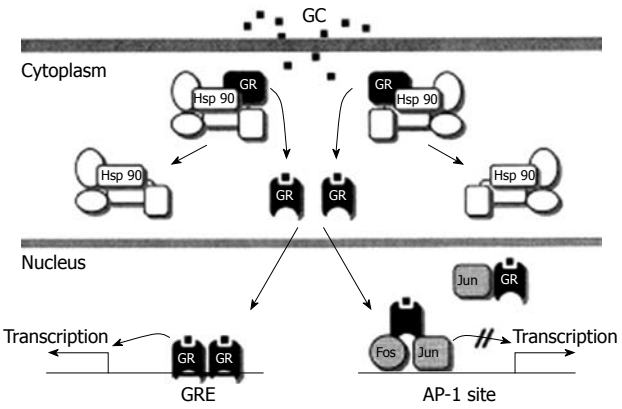

Figure 1 Simplified model of GR-mediated transcriptional modulation. (from Bamberger *et al* 1996).

also be involved in recruiting transport proteins to the receptor complex, regulating hormone binding to the receptor, and modulating the regulatory function of GR by disassembling transcription complexes[70-73].

Several other factors may influence GR-mediated transcriptional activation. For example, it has been suggested that competition between transcription factors for limited coactivator molecules may lead to gene repression[74,75]. Alternatively, cofactor effects may be restricted to designated compartments, and dynamic cofactor modules may hit promoters in a cyclic way during transcription[72-73,76-77]. In addition to acetylation, methylation and phosphorylation of histones may influence transcriptional control[78-80], as also could phosphorylation of the GR[81].

Some of the effects of GC occur too rapidly to be explained at the genomic level of transcription. Distinct GR forms or a cytosolic subset of GR may interact with signal transduction pathways, which are usually associated with membrane receptor signalling events[82]. Extensive crosstalk between steroid- and growth factor-stimulated signalling pathways occurs, and may impact the functioning of steroid receptors and genes that are regulated by steroids[83].

A number of synthetic GC have been developed with increased potency when compared to the naturally occurring substances. The increased potency, however, comes with an increased potential for adverse effects. The high potency of the synthetic steroid is attributed to its long half life and high affinity for the GR. DEX is commonly used in the research setting due to its' unique properties. The synthetic DEX differs from the naturally occurring cortisol due to the addition of a double bond between carbon 1 and 2, fluorination of the 9 position, and the addition of a methyl group. Therefore, while most GC in the plasma is bound to the CBG, DEX circulates freely, with blood concentrations unaffected by changes in CBG levels. This is particularly important in studies of development, as CBGs are known to be developmentally regulated[84]. The placental enzyme 11-β hydroxysteroid dehydrogenase type-2 (11β-HSD_2) protects the fetus by converting maternal cortisol into inactive cortisone, due to its dehydrogenase

activity[85]. However, the unique structure of DEX allows it to cross the placenta by escaping deactivation by 11β-HSD2.

Intestinal maturation

The effect of GC on prenatal intestinal maturation was investigated by Gartner *et al*[86], using mice lacking a functional glucocorticoid receptor (GR-/). Although a temporal association exists between the prenatal GC surge and corresponding intestinal development, the results of this study suggest that steroids are not mandatory for prenatal intestinal maturation. Histological examination of 18 d old fetal mice failed to demonstrate any effects of the GR-/- phenotype on intestinal morphology, or on measures of functional development (including enterocyte, Goblet and enteroendocrine cell numbers). LPH activity (as measured by X-gel staining) and the number of proliferating cells (as assessed by KI-67 staining) were also unaffected by the absence of GR. Thus, the rodent intestine appears to pass through a period of unresponsiveness to GC during fetal life, prior to becoming responsive in the postnatal period.

The role of GC in postnatal development has been well characterized. Indeed, adrenalectomy during the suckling period retards the functional maturation of the small intestine, while exogenous administration of GC may cause precocious maturation[87-89]. The mechanisms by which GC may influence maturation are not completely understood. There may be direct as well as indirect effects of GC. For example, Schaeffer *et al*[90]) injected hydrocortisone (50 g/g, *sc*) into 11 d old rats; they showed that the precocious induction of the BBM enzyme sucrase isomaltose (SI) paralleled decreases in TGF-β and the cytokine IL-1α, and increases in TNF-α. This finding suggests that cytokine levels may mediate the effects of GC on postnatal intestinal maturation.

The development of GC responsiveness was characterized in more detail by Solomon *et al*[89] using trehalase as a marker of intestinal maturation, they showed three phases of responsiveness to GC in mice: (1) a prenatal phase during which time Dexamethasone (DEX) did not induce maturation; (2) an early postnatal phase (first 2 postnatal weeks) of moderate responsiveness; and (3) a subsequent phase of increasing responsiveness in the third postnatal week. Postnatal increases in responsiveness were not paralleled by increases in GR abundance, but were associated with elevated circulating T4 concentrations. This observation fits with previous work demonstrating a synergistic effect of GC and T4 on BBM enzyme expression[91-93] also previously demonstrated three post-natal phases of responsiveness to GC. Based on the effects on SI mRNA and activity in rats, they identified (1) an early phase (day 10) when activation of the gene occurs; (2) a late phase (day 16) when changes in cell kinetics are observed; and (3) a loss of responsiveness (day 18) to GC. Although the exact timing of the various phases is not consistent between studies, it is clear that steroids have maximal effects in the early postnatal period.

Although some studies have shown that GC are not important in fetal development[86,89], not all researchers would agree with the suggestion that the fetus is unresponsiveness to GC. For example, Buchmiller and colleagues[94] showed that maternal DEX administration produced increases in small intestinal length and a trend towards an increase in LPH and maltase activity in fetal rabbits. The everted sleeve technique was used to demonstrate increases in glucose and proline transport in fetal rabbits exposed to DEX. When the fetal rabbits were subdivided into categories based on uterine position, runt fetuses exposed to DEX exhibited high nutrient uptake rates, surpassing the rates of the favored fetuses. This suggests that the runt intestine may be more responsive to steroid treatment, an observation with interesting implications regarding the potential clinical treatment of low birth weight or growth retarded infants.

The effects of GC on fetal intestine were also examined in a rabbit gastroschisis model. Gastroschisis is a herniation (displacement) of the intestines through a defect on one side of the umbilical cord. Intra-amniotic DEX (0.2 μg/g per day) infusion enhanced intestinal disaccharidase activity and glucose uptake in fetuses with experimentally induced herniation of the small intestine into the amniotic cavity[95]. While this study looks at a specific congenital dysfunction, it clearly demonstrates that the fetal intestine is capable of responding to GC.

What is the effect of GC on the intestine of human neonates? Nanthakumar *et al*[96]. used human intestinal xenografts to characterize the response of the developing intestine to GC. Responsiveness was determined by lactase activity and cytokine induction after a proinflammatory stimulus. Immature transplants (20 wk) responded to GC, but that this effect was lost in the mature (30 wk) transplants. This suggests that in humans there may also be a brief period of responsiveness *in utero* to GC.

Costalos *et al*[97] showed that in humans, maternal DEX administration increased fetal and neonatal plasma gastrin concentrations, while motilin was increased only in neonates, and vasointestinal peptide concentrations were unchanged. The authors speculate that the effects of GC on the gastrointestinal tract may be at least partially mediated by their actions on other GI hormones.

Clearly, there is contradictory evidence regarding the ability of the fetus to respond to steroids. The discrepancies may be due to the use of different indicators (trehalase, SI, nutrient uptake, morphology) of GC responsiveness. Maternal DEX administration may have a direct effect on the fetus, as DEX is known to cross the placenta[98]. However, it is also possible that the maternal administration of DEX has an indirect effect on the fetus, through the modulation of other factors such as cytokines, hormones or growth factors.

There are many other adverse effects of the therapeutic administration of GC, such as glaucoma,

poor wound healing, muscle atrophy, hypertension, hyperglycemia/diabetes, withdrawal syndrome, increased risk of infection, GI ulcers/perforation/bleed, thin skin, poorly developed muscle, thin extremities, fat collection in abdomen and upper back ("buffalo hump"), salt and water retention "moon face", osteoporosis, avascular necrosis, and CNS effects such as increased appetite, insomnia, euphoria and frank psychoses.

GC may also be used to accelerate lung maturation and surfactant production in premature infants, although DEX is not recommended as it is associated with impaired growth and neurodevelopmental delay. GC administration reduces the incidence of NEC in infants: a prospective experiment looking at prenatal or postnatal steroid use demonstrated a reduction in NEC in neonates[99]. Animal models of DEX administration demonstrate increased mucosal maturation, with increased enterocyte and goblet cells, coupled with a thinning of the muscularis[100]. Unfortunately, between early postnatal administrations of DEX is associated with the development of focal small bowel perforation[100,101]. The mechanism associated with the differential effect of GC on intestinal mucosa and muscularis is not fully understood. Although *in vitro* work on intestinal smooth muscle cells suggests that DEX alters the expression of collagen and other basement membrane elements[102,103], *in vivo* remodelling of the extracellular matrix by DEX has not been shown.

The tissue specific effects of GC may be the result of a redistribution of growth factors. DEX administration in newborn mice alters IGF- I immunolocalization, with increased protein detected in the mucosa, and reduced levels in the muscularis[104]. *In situ* hybridization analyses for IGF- I transcripts showed no differences in localization between the groups. In addition to its' effects on IGF- I immunolocalization, DEX (i.p. 1 μg/g, once daily) altered IGF- I binding protein composition in the mucosa in newborn mice. This alteration may be responsible for drawing IGF- I from the mesenchyme to the mucosa, and subsequently influencing intestinal maturation[105].

Further support for the involvement of the IGF- I system in NEC comes from the work of Burrin *et al*[106], who showed that in addition to decreases in circulating IGF- I and alterations in IGF- I binding proteins, neonatal pigs treated with DEX (subcutaneous, 1 mg/kg, for 7 d) had increased IGF- I receptor mRNA abundance. This increase was only observed in the stomach and ileum, with no effect seen in the jejunum. This corresponds well with the authors' observation that the effects of DEX are most pronounced in the ileum. The catabolic effects of DEX were also characterized in this study: small intestinal growth, particularly in the ileum, was inhibited due to increases in protein degradation, without significantly affecting protein synthesis.

GC have well documented effects on the ontogeny of BBM hydrolases. Adrenalectomy delays enzyme maturation during the third post-natal week, while exogenous GC administration may induce the precocious appearance of SI activity[107]. In adult animals, however, disaccharidase activities in the small intestine are unaffected by adrenalectomy or by GC administration[108]. The administration of DEX in the neonatal period increases the intestinal uptake of sugars (Drowdowski *et al*, unpublished observations, 2005).

There may be a syngeristic interaction between GC and T4 on BBM enzyme maturation. This was thought to involve the ability of T4 to increase corticosteroid-binding globulin, which subsequently reduces the clearance of hydrocortisone[109]. However, work by McDonald and Henning[110] demonstrated syngergism between DEX and T4. This indicates that an additional mechanism must be involved since DEX does not bind to CBG. In this study, animals received daily injections of subcutaneous T4 (130 pmol/g body weight) and a non-saturating dose of DEX (0.01 μg/g body weight) from post-natal day 5 to12. The hormones synergistically increased jejunal SI as well as ileal and duodenal alkaline phosphatase, and decreased ileal β-galactosidase activity, but did not affect jejunal or ileal LPH activity. When administered alone, T4 did not affect intestinal maturation, and DEX only partially stimulated maturation, when compared to the combination of hormones. The authors concluded that enzymes that rise post-natally responded to treatment with the combination of the T2 and DEX, while enzymes that decline post-natally show a mixed response.

In suckling rodents, GC regulate the expression of BBM enzymes such as SI-isomaltase and trehalase[107,111,112]. However, following GC administration, it takes 12-24 h to observe increases in the transcription of these genes. This delayed time-course suggests the involvement of secondary response genes[113]. Oesterreicher and Henning[114] identified a region of the trehalase promoter with potential binding sites for several transcription factors. Electromobility shift assays were performed using oligonucleotides from this region, as well as nuclear extracts from jejunum of 8 d old control or DEX-treated (1 g/g body wt) mice. They found that DEX stimulated expression of GATA-4 and GATA-6 proteins. These transcription factors are recognized as being important regulators of intestinal gene expression, and may interact with other transcription factors including those from the Sp family, Cdx, HNF-1 and HNF-4[115-120]. Indeed, GATA factors may allow cooperating transcription factors to bind to the DNA by altering the chromatin structure[119]. Although this study failed to prove that the induction of GATA factors leads to transcriptional activation of the BBM hydrolases, it does increase our understanding of possible mediators of GC effects.

Other molecular signals that may be responsible for the ontogenic changes in intestinal gene expression include a group of transcription factors called CCAAT/enhancer binding proteins (C/EBPs). In an attempt to determine regulatory mechanisms involved in the expression of the C/EBP α, β and δ isoforms, Boudreau *et al*[120] examined their expression in response to GC in the rat intestinal epithelial crypt-derived cell line

IEC-6, using Northern blot, transcription run-on assays, indirect immunofluorescence, Western blot, and electrophoretic mobility shift assays. Whereas C/EBP α expression was not regulated by GC, C/EBP β and δ mRNA and protein levels were rapidly induced. Moreover, C/EBP β- and δ-containing DNA binding complexes were increased by GC as determined by supershift assays, in contrast to C/EBP α containing complexes. Immunofluorescence studies showed cytoplasmic and nuclear localization for C/EBP α. This is in contrast to a restricted nuclear localization for both C/EBP β and C/EBP δ. Differential regulation by GC as well as the different localization of three C/EBP isoforms suggest a role for this class of transcription factors in the control of gene expression in intestinal epithelial cells.

Mechanisms of action

The mechanisms by which GC exert their effects may involve several other factors. Boudreau *et al*[121] showed that DEX increases c-fos and c-jun, and increased AP-1 DNA-binding capacity in IEC-6 cells. Ras transformation repressed the growth-inhibitory properties of DEX, and inhibited the induction of c-fos protein and mRNA. This suggests that Ras negatively modulates the response of intestinal epithelial cells to GC. Thiesen *et al*[122] have shown that early response genes may be involved in the effect of GC on the enhancement of intestinal absorption of nutrients that occurs after intestinal resection. This further illustrates the cross-talk that occurs between GC and intracellular signaling pathways.

GC may also exert their effects by influencing cellular proliferation, differentiation and apoptosis. Foligne *et al*[123] investigated the effects of 10-dbilateral adrenalectomy on morphometry, proliferation and apoptosis in the small intestine of 3 mo old Sprague-Dawley rats. Adrenalectomy led to partial atrophy and disorganization of the epithelium, with an increased number of goblet and Paneth cells. A reduction of crypt cell proliferation was paralleled by a marked increase in apoptosis in the villus.

Several other studies suggest that steroids may increase apoptosis. *In vitro* studies using rat jejunal epithelial cells (IEC-6) cells show that the locally acting budesonide increases apoptosis, while *in vivo* studies show that steroids increase apoptosis in intraepithelial lymphocytes[124,125].

There are conflicting reports on the effect of GC on intestinal proliferation. Low to medium doses (10^{-6}-10^{-11}) increase proliferation in IEC cells, while high doses (10^{-5}) inhibit proliferation[126]. Other studies suggest that the effect of steroids on proliferation depends on the developmental stage of the animal[127], Similarly, the location of the cells along the crypt-villous axis is an important factor as DEX reduced proliferation in the upper but not the lower crypt of fetal mouse duodenal explants[128]. This points to an important role of the adrenal glands and GC in the trophic status of the adult small intestinal mucosa. These results also highlight that the atrophy associated with GC is associated with a reduction in proliferation together with an increase in apoptosis. It is possible that some of the negative effects of GC on the intestine could be reduced or prevented by the administration of a trophic agent.

Early exposure to GC has been associated with lasting effects on cardiovascular, endocrine and metabolic systems. For example, prenatal glucocorticiod exposure results in reduced birth weight[129-134], increased blood pressure[135] increased expression of the glucocorticoid receptor in visceral fat[136], impaired coping in adverse situations[137], increases in corticosterone levels[135,137] hyperglycemia/hyperinsulinemia[131,138,139], increased susceptibility of the inner ear to acoustic noise trauma[140] and increases in PEPCK (rate-limiting enzyme in gluconeogenesis) mRNA and activity[141]. Steroids may also influence the development of the retina, although studies have shown both a positive effect on experimentally-induced retinopathy[142,143] and an increased risk of retinopathy of prematurity[144]. Furthermore, neurodevelopmental problems including an increased risk of cerebral palsy[145] have been documented following postnatal steroid administration.

It is not known if early exposure to GC results in lasting effects on intestinal function. However, because steroids are known to influence intestinal transport in adult animals[146], and because steroids influence the ontogeny of the intestine[110,147], it seems plausible that they may also play a role in the programming of intestinal function.

Effects on adult intestine

Does the adult small intestine remain responsive to steroids? Foligne *et al*[123] attempted to answer this question: three month old Sprague Dawley rats underwent bilateral adrenalectomies, and were sacrificed 10 d after surgery. Adrenalectomy modified maturation and differentiation, particularly in the proximal small intestine. A partial atrophy and disorganization of villous architecture was noted, coupled with decreases in crypt cell proliferation and increases in apoptotic cells in the upper villous region. LPH and SI were increased by adrenalectomy, while aminopeptidase N and intestinal alkaline phosphatase activities were reduced. These results indicate that the adrenal glands and GC play an important role in the trophic status of the small intestine in adult rats.

GC are used to treat various disorders including rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, allergies and asthma due to their anti-inflammatory effects[148]. Both NFκB and AP-1 are crucial for the induction of many genes involved in inflammation. GC may interfere with the transcriptional activity of these and other factors. Alternatively, GC may also interact with a negative GRE (nGRE) and thereby inhibit transcription. Although the anti-inflammatory effects of GC may be due to negative modulation of pro-inflammatory factors (transrepression), the side effects may be the result of transactivation[149]. This has created interest in developing "dissociating steroids", in which

the desirable transrepression effect is separated from undesirable transactivation effect.

Glucocorticoid resistance may occur as GC reduce GR expression[150]. The importance of GC is clear from the clinical symptoms associated with a deficiency or excess of the hormones. Cortisol deficiency (Addison's disease) is characterized by postural hypotension, weight loss and hypoglycaemia GC excess (Cushing's syndrome) is characterized by hypertension, central obesity and glucose intolerance.

Quaroni *et al*[151] found that exposing IEC cells to GC cause growth arrest, the formation of tight junctions, the appearance of tall slender microvilli, reorganization of the ER and Golgi, and decreased cdk6 as well as p^{27kip1} protein. These results are consistent with the activation of multiple genes important in the functioning of absorptive villous cells, but likely not involved in the induction of cell differentiation.

The role of the mesenchyme in mediating the effects of GR was studied by Simo *et al*[152] GC treatment of mesenchyme-derived cell populations resulted in an accumulation of laminin at the cell surface, accompanied by enhanced expression of BBM enzymes. This effect was abolished by anti-laminin, suggesting that GC may lead to accelerate laminin organization at the epithelial-mesenchymal interface, leading to epithelial cell differentiation.

Glucocorticosteroids and sugar transport

GC alter sugar transport in a number of other tissues. GLUT4 expression in adipose tissue is diminished in response to DEX, while GLUT4 in muscle is increased[153]. In adipocytes, DEX increased GLUT4 levels at the plasma membrane in the basal state, while GLUT4 translocation in response to insulin was inhibited.

Oral glucocorticosteroids increase intestinal sugar transport. For example, using an *in vivo* recirculation-perfusion technique in rats, Batt and Peters[154] showed that 7 d of prednisolone increased intestinal galactose absorption per enterocyte, without influencing the intestinal mucosa or cell kinetics. Similarly, short-term pharmacological doses of prednisolone increased digestive/absorptive function, while paradoxically decreasing the epithelial cell population in rats[155]. Long-term (28 d) administration of prednisolone or betamethasone in rats increased the activity of BBM proteins and galactose absorption, but induced atrophy of the mucosa and inhibition of cell turnover[156].

When the proximal jejunum of humans was perfused with glucose (28 mmol), intraluminal (100 mg/L) hydrocortisone increased sodium, water and glucose absorption[157] when compared to controls. Thiesen *et al*[146] assessed the influence of the glucocorticosteroids budesonide and prednisone on the *in vitro* uptake of sugars in weaning male rats. The steroids had no effect on the uptake of D-glucose by SGLT1. In contrast, the uptake of D-fructose by GLUT-5 was increased with both budesonide and prednisone. The increases in the uptake of fructose were not due to variations in the weight of the intestinal mucosa, food intake, or in GLUT-5 protein abundance or mRNA expression. This enhanced uptake of fructose was likely regulated by posttranslational processes, such as enhancement of the intrinsic activity of the transporters. There were no steroid-associated changes in mRNA expression of c-myc, c-jun, c-fos, proglucagon, or selected cytokines. However, the abundance of ileal ornithine decarboxylase mRNA was increased with Prednisone.

Thiesen *et al*[122] further characterized the effect of steroids using a model of intestinal resection. Adult male Sprague Dawley rats underwent transection or resection of 50% of the middle portion of the small intestine. Prednisone had no effect on the *in vitro* uptake of glucose or fructose in resected animals. In contrast, in resected rats budesonide increased by over 120% the value of the jejunal maximal transport rate (Vmax) for the uptake of glucose, and increased by over 150% ileal uptake of fructose. Changes in SGLT1, GLUT5, GLUT2, and Na^+K^+-ATPase protein abundance and mRNA expression did not explain the enhancing effect of budesonide. The steroids reduced c-jun, ODC and proglucagon expression. These data suggest that the influence of GC on sugar uptake in resected animals may be achieved by post-translational processes involving signalling with c-jun, ODC, and proglucagon, or perhaps also other as yet unknown signals.

GLUCAGON-LIKE PEPTIDE-2 (GLP2)

Biochemistry

Proglucagon is a 160 amino acid peptide encoded by the glucagon gene, and is present in intestinal L cells and α cells of the islets of Langerhans[213,214]. Proglucagon undergoes post-translational processing in the pancreas liberating glucagon as the main product. In the intestine, several peptide products (collectively referred to as "enteroglucagon") are produced including glucagon-like peptide-1 (GLP1) and glucagon-like peptide-2 (GLP2) (Figure 2).

Both GLP1 and GLP2 are secreted from the L cells of the distal small intestine and colon in response to enteral nutrients[215]. Both fatty acids[216] and glucose[217] stimulate secretion from L cells, but protein meals do not increase GLP1 or GLP2 secretion[218,219]. However, amino acid mixtures have been shown to stimulate GLP1 release in humans[220], and meat hydrolysates do stimulate GLP1 secretion from rat intestinal L cells *in vitro*[221].

The secretion of these peptides is biphasic, with an early peak within 30 min of a meal, followed by a later peak at 60-120 min[219]. It is thought that neuroendocrine pathways may be responsible for the early secretion, as luminal nutrients are not likely to reach the distal L cells within 30 min of ingestion[216,222].

The peptides are degraded in the plasma by dipeptidyl peptidase IV (DPPIV)[223], with a half-life for GLP2 of 7

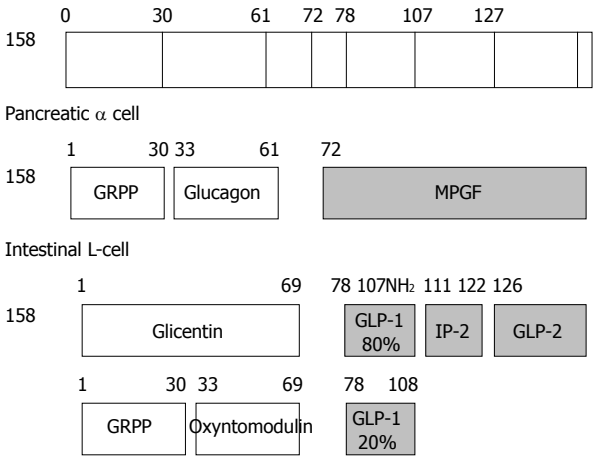

Figure 2 Post-translation processing of proglucagon in the pancreas and the intestinal L-cells. The numbers represent the amino acid at which enzymatic cleavage occurs.

min[224]. DPPIV-resistant analogs (ALX-600, Teduglutide) with greater potency have been developed for clinical use. These will be discussed in more detail in later sections.

The receptor is a G-protein coupled receptor with 7 transmembrane domains, and is encoded by a single gene localized to chromosome 17p13.3. GLP2R expression is highest in the proximal small intestine, and decreases distally along the longitudinal axis[225]. GLP2R has been localized to intestinal enteroendocrine cells in humans using immunohistochemistry[226], to enteric neurons in mice using reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry[227], and to rat enterocytes using ^{125}I-GLP2[228]. Orskov *et al*[229] found GLP2 receptors mainly on subepithelial myofibroblasts in rat, mouse, and human small and large intestine by immunohistochemistry and *in situ* hybridization. By double labelling they found that these GLP2 receptor immunoreactive cells also produce smooth muscle actin and keratinocyte growth factor (KGF). KGF antibody abolished the growth promoting effect of GLP2 in the large intestine, but not in the small intestine. This suggests that GLP2 in the gut may act by activating receptors on the subepithelial myofibroblasts, thereby causing the release of growth factors, which in turn stimulate intestinal growth. Therefore, at this time it remains unclear whether the intestinotropic effects of GLP2 are due to direct effects on enterocytes, or are mediated by secondary factors.

Physiological effects

While GLP1 has potent insulinotropic effects[230], GLP2 has been found to be an important intestinotrophic factor. The relationship between enteroglucagon and small bowel growth was first documented by Gleeson *et al*[231]: a patient with an enteroglucagon-producing tumor exhibited small bowel hyperplasia, and injection of the tumor extract into mice resulted in intestinal growth[232]. Drucker *et al*[233] demonstrated that GLP2 was the specific agent responsible for this effect. GLP2 appeared to exert a "tissue specific" effect on the gut, as no changes were found in other tissues including spleen, heart, brain and liver.

GLP2 given subcutaneously adult to mice (6-43 g, twice daily, for 10 d) increases small bowel weight and crypt-villous height[234,235]. Intestinal proliferation was increased, and apoptosis was reduced. The effect of GLP2 was not due to changes in food consumption. The effects of GLP2 were sustained, as increases in growth were still evident after three months of administration. The rapid turnover of the epithelium was thought to contribute to the lack of densensitization that was observed with prolonged GLP2 treatment.

In addition to its morphological effects, GLP2 (when administered intravenously or subcutaneously) also increases the activity and expression of BBM enzymes including SI, LPH, maltase-glucoamylase and aminopeptidase N[236-238]. Although the effects on gastric physiology in humans have been conflicting[239,240], inhibition of gastric emptying in pigs has been observed with GLP2 treatment[241]. Reductions in meal-stimulated gastric acid secretion have also been observed with GLP2 administration in humans[242]. GLP2 enhances barrier function in murine intestinal epithelium[243], making it a potential therapeutic for disorders such as inflammatory bowel disease and necrotizing pancreatitis, both of which are characterized by increases in intestinal permeability[243,244].

While the mechanism by which GLP2 stimulates the adaptive response is unknown, its effects on an immediate early gene, PC4/TIS7, have been observed. In postconfluent, quiescent IEC 18 cells in culture, a stable derivative of GLP2, r(gly2)GLP2, increased PC4/TIS7 expression. r(gly2)GLP2 administered intraperitoneally to mice similarly induced increased PC4/TIS7 mRNA compared with vehicle control[245].

A number of studies aimed at establishing the effect of GLP2 on intestinal transport. Cheeseman and Tsang[246] showed that a two hour vascular perfusion of GLP2 (4 h, 400 and 800 pmol/L) in rats increased D-glucose Vmax by about 65% in the baselateral membrane, and by three-fold in the BBM, with a concomitant increase in SGLT1 protein[247]. An anti-GLP antibody abolished the GLP2-induced increase in transport[246].

Not all studies have demonstrated an effect of GLP2 on nutrient transport. For example, Brubaker *et al*[237] treated mice subcutaneously with GLP2 (2.5 μg) for a 10 d period, and failed to reveal increases in the absorption of glucose or maltose. Curiously, an increased capacity for nutrient digestion was found to be due to increases in BBM enzyme activities, resulting in an apparent uncoupling of digestive capacity from subsequent sugar absorption. It is unclear if there is this discrepancy in the results of these studies on the effect of GLP2 or nutrient uptake.

The rapid trafficking the glucose and fructose transportation contributes to the sugar uptake GLUT2 into the BBM following a glucose infusion of meal[248-250]. GLUT2 protein levels in the BBM increased two-fold when luminal perfusions were increased from

0 mmol/L to 100 mmol/L glucose. One hour vascular infusion of GLP2 doubled the rate of fructose uptake into BBM following luminal fructose perfusion[251]. Fructose absorption in this study was determined by the appearance of fructose in the vascular bed. Western blotting of biotinylated surface-exposed protein showed a doubling of GLUT2 expression in the BBM following GLP2 infusion. Thus, GLP2 may promote the insertion of GLUT2 into the BBM, thereby providing a low affinity/ high capacity route in addition to SGLT1 and GLUTS by which fructose or glucose may be absorbed into the enterocyte.

Ramsanahie *et al*[252] examined the effect of chronically administered GLP2 on diurnal SGLT1 expression. Rats were treated with [Gly2]GLP2 (twice daily; 1 mg/g body weight) or vehicle (control) for 10 d. GLP2 administration did not alter the diurnal increase in mRNA levels of SGLT1, GLUT2, or GLUT5. However, SGLT1 protein was increased three-fold by GLP2, and *in situ* hybridization showed that SGLT1 mRNA was distributed along the entire length of the villi. This is in contrast to what was seen in control animals, where SGLT1 was restricted to the mid and upper villus, with less SGLT1 mRNA localized to the villous tip. Also, in contrast to the diffuse staining seen in control animals, immunofluorescence microscopy showed that SGLT1 protein in GLP2 treated animals was preferentially localized to the BBM, with little or no staining in the cytoplasm. This may represent another mechanism by which GLP2 increases intestinal glucose uptake.

The effect of GLP2 on *in vivo* nutrient absorption may also be attributed to nitric oxide-dependent increases in intestinal blood flow. Infusing GLP2 (500 pmol/kg per hour) for 4 h in TPN-fed piglets led to increased portal-drained visceral (PDV) blood flow rate, intestinal blood volume, and PDV glucose uptake. GLP2 also increased intestinal constitutive nitric oxide synthase (NOS) activity and endothelial NOS protein abundance[253]. Thus, in TPN-fed neonatal pigs, GLP2 acutely stimulates intestinal blood flow and glucose utilization, and that this response is nitric oxide-dependent.

Walsh *et al*[254] studied GLP2 signalling in isolated rat intestinal mucosal cells expressed mRNA transcripts for the GLP2R, as well as for chromogranin A and β-tubulin Ⅲ, markers for enteroendocrine and neural cells, respectively. cAMP production in response to [Gly2]GLP2, a degradation-resistant analog of GLP2, was maximal at 10^{-11} mol/L with reduced cAMP accumulation observed at higher doses. The cAMP response was abolished by pretreatment with 10^{-6} mol/L GLP2, indicating receptor desensitization. GLP2 treatment of isolated mucosal cells increased ^{3}H-thymidine incorporation, and this was prevented by inhibition of the protein kinase A pathway. In contrast, GLP2 did not affect p44/p42 MAPK phosphorylation or the levels of cytosolic calcium in the mucosal cell preparation. These results provide evidence that activation of the endogenous rat mucosal GLP2 receptor is linked to activation of a cAMP/protein kinase A-dependent, growth-promoting pathway *in vitro*.

Estall *et al*[255] examined the mechanisms regulating signaling, internalization, and trafficking of the GLP2R to identify determinants of receptor activation and desensitization. Heterologous cells expressing the transfected rat or human GLP2R exhibited a rapid, dose-dependent, and prolonged desensitization of the GLP2-stimulated cAMP response and a sustained GLP2-induced decrease in levels of cell surface receptor. Surprisingly, inhibitors of clathrin-dependent endocytosis failed to significantly decrease GLP2R internalization, whereas cholesterol sequestration inhibited ligand-induced receptor internalization and potentiated homologous desensitization. The hGLP2R localized to both Triton X-100-soluble and -insoluble (lipid raft) cellular fractions and colocalized transiently with the lipid raft marker caveolin-1. Although GLP2R endocytosis was dependent on lipid raft integrity, the receptor transiently associated with green fluorescent protein tagged-early endosome antigen 1-positive vesicles and inhibitors of endosomal acidification attenuated the reappearance of the GLP2R on the cell surface. This data demonstrates that GLP2R desensitization and raft-dependent trafficking represent distinct and independent cellular mechanisms and provide new evidence implicating the importance of a clathrin- and dynamin-independent, lipid raft-dependent pathway for homologous G protein-coupled receptor internalization.

Intestinal resection

Plasma GLP2 levels rise following intestinal resection in rats[256-258]. When rats were subjected to a 70% midjejunoileal resection or ileal transection, and were maintained with TPN or oral feeding. Resection-induced adaptive growth in TPN- and orally-fed rats was associated with a significant positive correlation between increases in plasma bioactive GLP2 and proglucagon mRNA abundance in the colon of TPN-fed rats and in the ileum of orally fed rats[259]. While these increases were transient in the TPN-fed group, luminal nutrients produced a sustained increase detected at 3 and 7 d post-resection. These data support a significant role for endogenous GLP2 in the adaptive response to mid-small bowel resection in both TPN and orally fed rats.

GLP2 administration in rats increases the adaptive response to massive intestinal resection[260]. Sprague-Dawley rats were divided into two groups, with a 75% mid-jejunum-ileum resection and a sham operated group. Animals were given 0.1 μg/g GLP2 analog (protease resistant human GLP2) or placebo given subcutaneously twice daily for 21 d. Administration of the GLP2 analog was associated with an increase of the mucosal mass in the proximal jejunum and terminal ileum.

Martin *et al*[261] investigated the effects of GLP2 in a total parenteral nutrition (TPN)-supported model of experimental short bowel syndrome. Juvenile Sprague-Dawley rats underwent a 90% small intestinal resection, and were randomized to three groups: enteral diet and intravenous saline infusion, TPN only, or TPN +

10 μg/kg per hour GLP2. TPN plus GLP2 treatment resulted in increased bowel and body weight, villos height, intestinal mucosal surface area, crypt cell proliferation, and reduced intestinal permeability, as compared with the TPN alone animals. GLP2 increased serum GLP2 levels and intestinal SGLT-1 protein abundance as compared with either TPN or enteral groups. This demonstrates that GLP2 is capable of stimulating intestinal adaptation in the absence of enteral feeding. Because a number of hormones and growth factors have been shown to influence intestinal function, Washizawa *et al*[262] compared the effects of GLP2, growth hormone (GH) and keratinocye growth factor (KGF) on markers of gut adaptation following massive small bowel resection (MSBR). KGF increased goblet cell numbers and TTF3, a cytoprotective trefoil peptide, in both the small bowel and the colon. While both GH and KGF increased colonic mucosal growth, GLP2 exerted superior trophic effects on jejunal growth. GLP2 also increased the glutathione/glutathione disulfide ratio, resulting in improved mucosal glutathione redox status throughout the bowel. Because of the differential effects of GLP2, GH and KGF on gut adaptation following MSBR, the authors conclude that a combination of these agents may be most beneficial.

Human studies with GLP2 have also been performed: a non-placebo controlled study was conducted in 8 patients with short bowel syndrome (SBS) with an end-enterostomy type of anastomasis (6 had Crohn's disease and 4 were not receiving HPN)[263]. Treatment with GLP2 (400 μg subcutaneously twice a day for 35 d) increased intestinal absorption of energy, body weight, and lean body mass. Crypt depth and villous height were also increased in 5 and 6 patients, respectively.

The results of more recent studies of GLP2 in SBS have been reviewed[264] and concluded that "Currently, hormonal therapy in short-bowel patients should be considered experimental and it is only recommended in research studies. The optimal duration and concentration requirements for GLP2 to induce beneficial effects on intestinal secretion, motility, morphology and most importantly absorption, are not known. Optimal dosage and administration of this new treatment to short-bowel patients may eventually result in long-term improvements in nutritional status and independence of parenteral nutrition in a larger fraction of short-bowel patients".

Intestinal development

The topic of the Development of the Infant Intestine: Implications for Nutrition Support and a consideration of trophic factors essential to intestinal development, have recently been considered[265]. A role for GLP2 in the ontogeny of the intestine has been proposed. Lee *et al*[266] established that proglucagon mRNA was detectable in the rat fetus, and that immunoreactivity increased in the early neonatal period. Prohormone convertases, which are required for the liberation of GLP2 from proglucagon, are also detected in the fetal rat. Lovshin *et al*[267] detected GLP2R mRNA during fetal and neonatal development in the rat, with levels being higher in the fetal and neonatal gut as compared to adult rats. High levels of GLP2 (1-33) were also detected in the circulation of 13 d old neonatal rats, and GLP2 immunoreactivity was found in the fetal rat intestine. In order to prove that fetal cells were capable of secreting GLP2, fetal rat intestinal cell cultures were studied, and were found to secrete correctly processed GLP2 (1-33). The administration of a degradation resistant GLP2 analog [h (Gly2)-GLP2] to 1 d old rat pups for a period of 10 d resulted in increases in both small bowel weight and length. Thus, the GLP2/GLP2R axis is functional in early life, and that the developing intestine is capable of synthesizing, secreting and responding to GLP2.

A subsequent study done by Petersen *et al*[236] on premature (92% gestation) TPN fed piglets demonstrated that in addition to increases in maltase mRNA and activity, increases in SI and aminopeptidase N activities were observed. Thus, it appears that some of the effects that GLP2 exerts on intestinal function may be related to gestational age at birth, as the premature intestine was more responsive to exogenous GLP2 than the term neonatal intestine. The authors also noted that GLP2 infused into pig fetuses *in vivo* passed into the maternal circulation[268]. This suggests that GLP2 may pass through the placenta, and conversely, may expose the fetus to maternal GLP2. This raises the possibility that GLP2 given to pregnant animals may alter the form and function of the offspring.

Signalling events

Even before the GLP2 receptor was cloned, the role of the PI3K pathway in mediating the effects of GLP2 on intestinal sugar transport was studied by Cheeseman *et al*[247]: *in vivo* infusions of GLP2 produced an acceleration of sodium-dependent glucose uptake into BBM vesicles, with similar increase in SGLT-1 abundance. The effect of GLP2 could be inhibited by luminal brefeldin A, which blocks protein trafficking from the Golgi to the plasma membrane, or by the PI3K inhibitor, wortmannin. These results indicate that GLP2 is able to induce trafficking of SGLT-1 from an intracellular pool into the BBM and that PI3K may be involved in the intracellular signaling pathway in this response.

Because the GLP2R is not expressed on any intestinal cell lines, *in vitro* studies on GLP2 receptor signalling have been carried out in transfected heterologous cell types. Work done on transfected baby hamster kidney fibroblasts showed that GLP2 stimulated AP-1 dependent pathways increased cAMP, but did not change intracellular calcium levels[269]. There is decreased apoptosis and reduced caspase-3 activation following GLP2 treatment *in vitro*[270]. PKA, PI3K and the ERK pathways were not found to be essential for GLP2 inhibition of apoptosis, which was associated with reductions in cytochrome c release and cleavage of poly ADP-ribose polymerase (PARP).

GLP2-treated cells (10 μm, 3 d) demonstrated a greater than 10-fold increase in proliferation in Caco2

cells[271]. This response was inhibited by PI3K and mitogen activated/extracellular signal-regulated kinase (MEK) inhibitors. A significantly greater abundance of the phosphorylated forms of both ERK-1 and ERK-2 was present in cells following GLP2. This suggests that the increase in Caco-2 proliferation in response to GLP2 may be due, at least in part, to the involvement of both the PI 3-kinase and the MAPK pathways.

The limitations of this work, however, centers around the fact that both the authors of this study as well as Yusta *et al*[270] were unable to show, using Western blotting or RT-PCR, that Caco2 cells express endogenous GLP2R. This suggests that the proliferative effect of GLP2 on Caco2 cells may be mediated by other receptors, such as the EGFR, which mediates GLP1 induced proliferation in pancreatic β cells[272]. Furthermore, the observation that GLP2 is able to induce proliferation in a tumor cell lines, raises a concern regarding the role of GLP2 in promoting tumor growth. Indeed, recent work by Thulesen *et al*[273] showed that GLP2 promotes the growth of mucosal neoplasms in female C57bl mice whose colonic tumours were experimentally induced by administering a methylating carcinogen.

Koehler *et al*[274] identified several expressed sequence tags from human cervical carcinoma cDNA libraries that correspond to GLP2R nucleotide sequences. GLP2R mRNA transcripts were detected by RT-PCR in HeLa cervical carcinoma cells and Ca Ski cervical carcinoma cells. GLP2 increased cAMP accumulation and activated ERK1/2 in HeLa cells transiently expressing the cloned human HeLa cell GLP2R cDNA. However, the GLP2R-induced activation of ERK1/2 was not mediated through Gαs, adenylyl cyclase, or transactivation of the epidermal growth factor receptor, but was pertussis toxin sensitive, inhibited by dominant negative Ras, and dependent on betagamma-subunits. GLP2 also induced a significant increase in bromodeoxyuridine incorporation that was blocked by dominant negative Ras. Furthermore, GLP2 inhibited HeLa cell apoptosis induced by LY294002 in a protein kinase A-dependent, but ERK-independent, manner. These findings demonstrate that the HeLa cell GLP2R differentially signals through both G(α)s/cAMP- and G(i)/G(o)-dependent pathways, illustrating for the first time that the GLP2R is capable of coupling to multiple heterotrimeric G proteins defining distinct GLP2R-dependent biological actions.

Finally, the effects of GLP2 may be due to transactivation of other cell surface receptors. For example, GLP1 increases PI3K activity and enhances β-cell proliferation via transactivation of the EGFR[272].

Yusta *et al*[275] demonstrated that GLP2, in a cycloheximide-insensitive manner, enhanced survival in baby hamster kidney cells stably transfected with the rat GLP2R, reduced mitochondrial cytochrome c efflux, and attenuated the caspase-dependent cleavage of Akt, poly (ADP-ribose) polymerase, and β-catenin following inhibition of phosphatidylinositol 3-kinase (PI3K) by LY294002. The prosurvival effects of GLP2 on LY294002-induced cell death were independent of Akt, p90 (Rsk), or p70 S6 kinase activation; were mimicked by forskolin; and were abrogated by inhibition of protein kinase A (PKA) activity. GLP2 inhibited activation of glycogen synthase kinase-3 (GSK-3) through phosphorylation at Ser (21) in GSK-3α and at Ser (9) in GSK-3β in a PI3K-independent, PKA-dependent manner. GLP2 reduced LY294002-induced mitochondrial association of endogenous Bad and Bax and stimulated phosphorylation of a transfected Bad fusion protein at Ser (155) in a PI3K-independent, but H89-sensitive manner, a modification known to suppress Bad pro-apoptotic activity. These results suggest that GLP2R signaling enhances cell survival independently of PI3K/Akt by inhibiting the activity of a subset of pro-apoptotic downstream targets of Akt in a PKA-dependent manner.

Rocha *et al*[276] assessed the proliferative actions of GLP2 on the human Caco-2 cell line. GLP2 stimulated proliferation was inhibited in a dose-dependent fashion by both pertussis and cholera toxin (specific G protein inhibitors). This suggests that a G-protein-linked signaling pathway is involved with GLP2 bioactivity in Caco-2 cells. GLP2 stimulated proliferation was also augmented by 2',5'-dideoxyadenosine, which increases adenylate cyclase. Proliferation rates were inversely proportional to changes in intracellular cAMP concentration. These findings suggest that a G-protein linked signaling pathway is involved with GLP2 bioactivity in the intestinal epithelial cell line Caco-2.

Clinical

Burrin *et al*[277] studied 38 TPN-fed neonatal piglets infused intravenously with either saline or GLP2 at three rates (2.5, 5.0, and 10.0 nmol/kg per day for 7 d). GLP2 infusion dose-dependently increased small intestinal weight, DNA and protein content, and villus height; however, stomach protein synthesis was decreased by GLP2. Intestinal crypt and villus apoptosis decreased and crypt cell number increased linearly with GLP2 infusion rates, whereas cell proliferation and protein synthesis were stimulated only at the high GLP2 dose. The intestinal activities of caspase-3 and -6 and active caspase-3 abundance decreased, yet procaspase-3 abundance increased markedly with increasing infusion rate and plasma concentration of GLP2. The GLP2-dose-dependent suppression of intestinal apoptosis and caspase-3 activity was associated with increased protein kinase B and glycogen-synthase kinase-3 phosphorylation, yet the expression phosphatidylinositol 3-kinase was unaffected by GLP2. Intestinal endothelial nitric oxide synthase mRNA and protein expression was increased, but only at the high GLP2 dose. These authors concluded that the stimulation of intestinal epithelial survival is concentration-dependent at physiological GLP2 concentrations. However, induction of cell proliferation and protein synthesis is a pharmacological response. Moreover, they showed that GLP2 stimulates intestinal cell survival and proliferation

in association with induction of protein kinase B and glycogen-synthase kinase-3 phosphorylation and Bcl-2 expression.

Thus, GLP2 increases intestinal growth in premature, TPN-fed pigs by decreasing proteolysis and apoptosis, and enteral nutrition was not required for these effects to occur. The actions of GLP2 are transduced by the GLP2 receptor (GLP2R), which was characterized and cloned by Munroe *et al*[225]. GLP2R was detected in several rat tissues including the stomach, small bowel, colon, and in small quantities in other tissues such as brain and lung[225]. This raises the possibility that GLP2 may have effects beyond the small intestine. Indeed, Haderslev *et al*[278] found that GLP2 administration significantly increased spinal bone mineral density in short-bowel patients with no colon, due to beneficial effects of GLP2 on bone resorption[279]. Centrally administered GLP2 increases satiety in rodents[280,281], while peripherally administered GLP2 does not influence gastric emptying, food intake or satiety in humans[238,282].

Although the effects of early exposure to hormones such as GLP2 are unknown, the presence of circulating GLP2 and the detection of the GLP2R in fetal and neonatal rats[267] suggests a role for GLP2 in regulating intestinal development. Drozdowski *et al* (unpublished observations, 2006) has shown that GLP2 modifies intestinal morphology when given to suckling rats, and enhances sugar uptake into the intestine of rats whose mothers were given GLP2 during pregnancy and lactation. Therefore, it is possible that exposing young animals to GLP2, either directly or *via* their pregnant and lactating dams, may also have lasting effects on intestinal function.

GLP2 and glucocorticosteroids

The possibility of a growth factor or hormone acting additively or synergistically with a steroid hormone has been demonstrated in various tissues. For example, EGF potentiates the proliferative effects of progesterone and estrogen in the mammary gland[283]. In a review, Lange[83] discusses the cross-talk that occurs between steroid hormone receptors and intracellular signalling pathways. There is clearly evidence of non-genomic and extra-nuclear functions of steroid receptors, including the initiation of signal transduction pathways. Indeed, this type of cross-talk may explain how genes are coordinately regulated by mitogenic stimuli in hormone responsive tissues. For example, Migliaccio *et al*[284] reported MAPK activation by estradiol, and interactions between the progesterone receptor, the estrogen receptor and p60-Src kinase.

A synergistic effect between DEX and GLP2 may occur in the intestine, as GC have a permissive effect on several hormones including catecholamines, thyroid hormones, growth hormone and ACTH[285]. GC mediates a permissive action, mostly for hormones, which act on G-protein coupled receptors, and increases adenylate cyclase[286,287]. This occurs because DEX alters adenylyl cyclase, enhancing the effects of cAMP generating agonists. Therefore, an interaction between DEX and GLP2 is likely, as the GLP2 receptor is a G-protein coupled receptor.

Furthermore, cAMP and PKA, which are activated by GPCRs like GLP2R, may increase the steroid sensitivity of a target cell by increasing the DNA binding ability of the GR for its response elements. In a study by Rangarajan *et al*[288] using embryonal carcinoma cells lacking cAMP response element binding protein (CREB), activation of PKA increased hormone-dependent trans-activation of the GR. The effect of PKA was related to the DNA binding domain of the GR, as deletion of the amino-terminal or the ligand-binding domain did not alter PKA's effect. However, the absence of a consensus PKA phosphorylation site within the GR DNA binding domain led the authors to suggest that the GR is not a direct substrate for phosphorylation by PKA. Instead, they propose a multistep process involving other cellular kinases and phosphatases that may interact with the GR.

As discussed previously, there are several pieces of evidence that suggest that GLP2 may exert its effect *via* the PI3K pathway. Steroid receptors may interact with this pathway as Simoncini *et al*[82] showed that the estrogen receptor binds to p85 subunit of PI3K.

Although there are no reports of the effect of DEX on PI3K in the intestine, an association has been observed in other tissues. For example, Saad *et al*[289] showed that DEX induced a 69% increase in the level of PI 3-kinase in adipocytes as determined by immunoblotting. Conversely, Buren *et al*[290] demonstrated that DEX decreases PI3K, PKB, insulin-stimulated PKB phosphorylation and glucose transport in isolated rat adipocytes, without changes in GLUT4. These results suggest that glucocorticoids, independently of the surrounding glucose and insulin concentration, impair glucose transport capacity in fat cells. Finally, Krasil'nikov *et al*[291] showed that prolonged exposure to DEX increased PI3K in Rous sarcoma virus-transformed hamster fibroblasts. Certainly in other tissues, such as adipose and muscle, PI3K is involved in regulating insulin-stimulated glucose uptake[292]. In the intestine, EGF stimulated increases in intestinal glucose transport in rabbits is abolished by the PI3K inhibitor LY294002[293]. Similarly, IGF- I associated increases in jejunal glucose uptake and Na^+K^+-ATPase activity are abolished by wortmannin, another PI3K inhibitor[23]. So clearly this pathway plays an important role in transducing signals from hormones and growth factors to the proteins involved in sugar transport. Indeed, preliminary studies in the young rat show that GLP2 and DEX may stimulate the intestinal uptake of glucose, and this is essential with increased abundance of Akt and mTOR, part of the PI3K pathway.

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REVIEW

Intussusception of the bowel in adults: A review

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Abstract

Intussusception of the bowel is defined as the telescoping of a proximal segment of the gastrointestinal tract within the lumen of the adjacent segment. This condition is frequent in children and presents with the classic triad of cramping abdominal pain, bloody diarrhea and a palpable tender mass. However, bowel intussusception in adults is considered a rare condition, accounting for 5% of all cases of intussusceptions and almost 1%-5% of bowel obstruction. Eight to twenty percent of cases are idiopathic, without a lead point lesion. Secondary intussusception is caused by organic lesions, such as inflammatory bowel disease, postoperative adhesions, Meckel's diverticulum, benign and malignant lesions, metastatic neoplasms or even iatrogenically, due to the presence of intestinal tubes, jejunostomy feeding tubes or after gastric surgery. Computed tomography is the most sensitive diagnostic modality and can distinguish between intussusceptions with and without a lead point. Surgery is the definitive treatment of adult intussusceptions. Formal bowel resection with oncological principles is followed for every case where a malignancy is suspected. Reduction of the intussuscepted bowel is considered safe for benign lesions in order to limit the extent of resection or to avoid the short bowel syndrome in certain circumstances.

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Key words: Intussusception; Intestinal invagination; Adult; Bowel obstruction; Computed tomography; Ultrasonography; Surgery

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INTRODUCTION

First reported in 1674 by Barbette of Amsterdam[1] and further presented in a detailed report in 1789 by John Hunter[2] as "introssusception", intussusception represents a rare form of bowel obstruction in the adult, which is defined as the telescoping of a proximal segment of the gastrointestinal (GI) tract, called intussusceptum, into the lumen of the adjacent distal segment of the GI tract, called intussuscipiens. Historically, Sir Jonathan Hutchinson was the first to operate on a child with intussusception in 1871[3].

Adult intussusception represents 5% of all cases of intussusception and accounts for only 1%-5% of intestinal obstructions in adults[4]. The condition is distinct from pediatric intussusception in various aspects. In children, it is usually primary and benign, and pneumatic or hydrostatic (air contrast enemas) reduction of the intussusception is sufficient to treat the condition in 80% of the patients. In contrast, almost 90% of the cases of intussusception in adults are secondary to a pathologic condition that serves as a lead point, such as carcinomas, polyps, Meckel's diverticulum, colonic diverticulum, strictures or benign neoplasms, which are usually discovered intraoperatively[5-7]. Due to a significant risk of associated malignancy, which approximates 65%[8,9], radiologic decompression is not addressed preoperatively in adults. Therefore, 70 to 90% of adult cases of intussusception require definite treatment, of which surgical resection is, most often, the treatment of choice[10].

MECHANISM-PATHOPHYSIOLOGY

In adults, the exact mechanism of bowel intussusception

is unknown (primary or idiopathic) in 8%-20% of cases and is more likely to occur in the small intestine[4,10,11]. On the other hand, secondary intussusception is believed to initiate from any pathologic lesion of the bowel wall or irritant within the lumen that alters normal peristaltic activity and serves as a lead point, which is able to initiate an invagination of one segment of the bowel into the other[10,12]. Schematically, intussusception could be described as an "internal prolapse" of the proximal bowel with its mesenteric fold within the lumen of the adjacent distal bowel as a result of overzealous or impaired peristalsis, further obstructing the free passage of intestinal contents and, more severely, compromising the mesenteric vascular flow of the intussuscepted segment. The result is bowel obstruction and inflammatory changes ranging from thickening to ischemia of the bowel wall.

LOCATION-ETIOLOGY

The most common locations in the gastrointestinal tract where an intussusception can take place are the junctions between freely moving segments and retroperitoneally or adhesionally fixed segments[13]. Intussusceptions have been classified according to their locations into four categories: (1) entero-enteric, confined to the small bowel, (2) colo-colic, involving the large bowel only, (3) ileo-colic, defined as the prolapse of the terminal ileum within the ascending colon and (4) ileo-cecal, where the ileo-cecal valve is the leading point of the intussusception and that is distinguished with some difficulty from the ileo-colic variant[5,8,14].

Intussusceptions have also been classified according to etiology (benign, malignant or idiopathic). In the small intestine, an intussusception can be secondary either to the presence of intra- or extra-luminal lesions (inflammatory lesions, Meckel's diverticulum, postoperative adhesions, lipoma, adenomatous polyps, lymphoma and metastases) or iatrogenic, e.g. due to the presence of an intestinal tube[15] or even in patients with a gastrojejunostomy[16]. Malignancy (adenocarcinoma) accounts for up to 30% of cases of intussusception occurring in the small intestine[10]. A very rare case from our department's experience was a 29-year old male patient with a diffuse, small B-cell (Burkitt-like) non-Hodgkin lymphoma of the ileum who developed an ileo-colic intussusception. On the other hand, intussusception occurring in the large bowel is more likely to have a malignant etiology and represents up to 66% of the cases[10,12,17].

CLINICAL PRESENTATION

The clinical presentation of adult intussusception varies considerably. The presenting symptoms are nonspecific and the majority of cases in adults have been reported as chronic, consistent with partial obstruction[4,18]. The classic pediatric presentation of acute intussusception (a triad of cramping abdominal pain, bloody diarrhea and a palpable tender mass) is rare in adults. Nausea, vomiting, gastrointestinal bleeding, change in bowel habits, constipation or abdominal distension are the nonspecific symptoms and signs of intussusception[5,8].

Intussusception in adults can be further classified according to the presence of a lead point or not[19]: transient non-obstructing intussusception without a lead point has been described in patients with celiac[20] or Crohn's[21] disease, but is more frequently idiopathic and resolves spontaneously without any specific treatment. On the other hand, intussusception with an organic lesion as the lead point usually presents as a bowel obstruction, persistent or relapsing, necessitating, however, a definite surgical therapy.

DIAGNOSIS-IMAGING

Variability in clinical presentation and imaging features often make the preoperative diagnosis of intussusception a challenging and difficult task. Reijnen *et al*[22] reported a preoperative diagnostic rate of 50%, while Eisen *et al*[17] reported a lower rate of 40.7%.

Plain abdominal films are typically the first diagnostic tool, since in most cases the obstructive symptoms dominate the clinical picture. Such films usually demonstrate signs of intestinal obstruction and may provide information regarding the site of obstruction[17,23]. Upper gastrointestinal contrast series may show a "stacked coin" or "coil-spring" appearance, while a barium enema examination may be useful in patients with colo-colic or ileo-colic intussusception, during which a "cup-shaped" filling defect or "spiral" or "coil-spring" appearances are characteristically demonstrated[17,24,25].

Ultrasonography is considered a useful tool for the diagnosis of intussusception, both in children and in adults[26,27]. The classical imaging features include the "target" or "doughnut" signs on the transverse view and the "pseudo-kidney" sign or "hay-fork" sign in the longitudinal view[27,28]. Undoubtedly, this procedure requires handling and interpretation by an experienced radiologist, in order to confirm the diagnosis. However, obesity and the presence of massive air in the distended bowel loops limit the image quality and the subsequent diagnostic accuracy.

Abdominal computed tomography (CT) is currently considered as the most sensitive radiologic method to confirm intussusception, with a reported diagnostic accuracy of 58%-100%[4,12,29-33]. The characteristic features of CT scan include an unhomogeneous "target" or "sausage"- shaped soft- tissue mass with a layering effect (Figure 1); mesenteric vessels within the bowel lumen are also typical[10]. A CT scan may define the location, the nature of the mass, its relationship to surrounding tissues and, additionally, it may help staging the patient with suspected malignancy causing the intussusception[17]. In a recent interesting report by Kim *et al*[19], abdominal CT was able to distinguish between intussusception without a lead point (features: no signs of proximal bowel obstruction, target-like or sausage-shaped mass, layering effect) from that with a lead point (features: signs of bowel obstruction, bowel wall edema with loss of the classic three-layer appearance due to

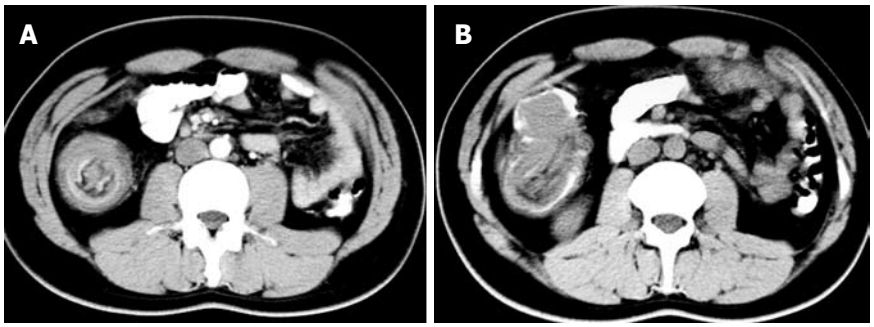

Figure 1 Abdominal computed tomography in adult intussusception. A: The characteristic "target"-shaped soft-tissue mass with a layering effect of a 29-year old male patient with a diffuse, small B-cell (Burkitt-like) non Hodgkin lymphoma of the ileum who developed an ileo-colic intussusception; B: A "sausage"-shaped soft tissue mass in the ascending colon of the same patient.

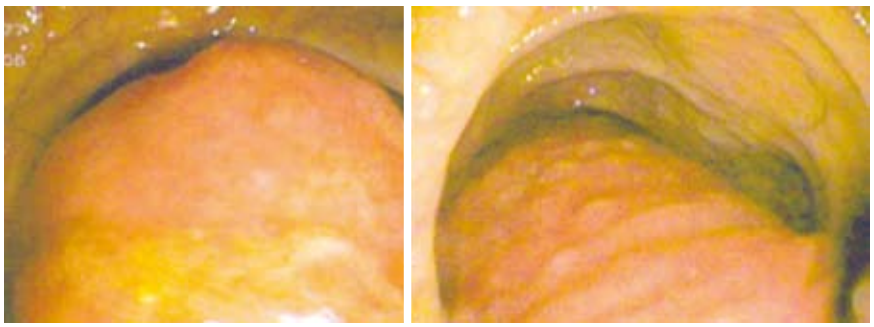

Figure 2 Colonoscopy. Revealing the presence of the inverted terminal ileum (intussusceptum) in the ascending colon (intussuscipiens) in a patient with an ileo-cecal intussusception due to an ileal lipoma.

Figure 3 Intraoperative findings. A: Thickened, congested and inflamed terminal ileum with proximal small bowel obstruction in a 75-year old woman with ileo-colonic intussusception; B: The surgical specimen after the en bloc resection of the terminal ileum and the ascending colon in the same patient; C: The cause of the intussusception was a lipoma of the ileo-cecal valve (arrow).

impaired mesenteric circulation and demonstration of the lead mass), and this may help reducing the number of unnecessary surgical interventions.

DIAGNOSIS-ENDOSCOPY

Flexible endoscopy of the lower GI tract is considered invaluable in evaluating cases of intussusception presenting with subacute or chronic large bowel obstruction[10]. Confirmation of the intussusception, localization of the disease and demonstration of the underlying organic lesion serving as a lead point are the main benefits of endoscopy. Snare polypectomy is not advisable in individuals with chronic intussusception presenting with a polypoid mass on barium or endoscopic examination, due to the high risk of perforation occurring in a background of chronic tissue ischemia and possible necrosis of the intussuscepted bowel segment's wall[34,35]. In the case of a lipoma as a lead point of an intussusception (Figure 2), typical colonoscopic features include a smooth surface, the "cushion sign" or pillow sign" (forcing the forceps against the lesion results in depression of the mass) and the "naked fat sign" (fat extrusion during biopsy)[36-38].

SURIGICAL TREATMENT

Due to the fact that adults present with acute, subacute, or chronic nonspecific symptoms[9], the initial diagnosis is missed or delayed and is established only when the patient is on the operating table (Figure 3). Most surgeons accept that adult intussusception requires surgical intervention because of the large proportion of structural anomalies and the high incidence of occurring malignancy. However, the extent of bowel resection and the manipulation of the intussuscepted bowel during reduction remain controversial[10]. In contrast to pediatric patients, where intussusception is primary and benign, preoperative reduction with barium or air is not suggested as a definite treatment for adults[10,17,39].

The theoretical risks of preliminary manipulation and reduction of an intussuscepted bowel include: (1) intraluminal seeding and venous tumor dissemination,

(2) perforation and seeding of microorganisms and tumor cells to the peritoneal cavity and (3) increased risk of anastomotic complications of the manipulated friable and edematous bowel tissue[4,5,10,17,22]. Moreover, reduction should not be attempted if there are signs of inflammation or ischemia of the bowel wall[33]. Therefore, in patients with ileo-colic, ileo-cecal and colo-colic intussusceptions, especially those more than 60 years of age, due to the high incidence of bowel malignancy as the underlying etiologic factor, formal resections using appropriate oncologic techniques are recommended, with the construction of a primary anastomosis between healthy and viable tissue[8,10,17,22,38,40]. Azar *et al*[4] report that, for right-sided colonic intussusceptions, resection and primary anastomosis can be carried out even in unprepared bowels, while for left-sided or rectosigmoid cases resection with construction of a colostomy and a Hartmann's pouch with re-anastomosis at a second stage is considered safer, especially in the emergency setting.

However, when a preoperative diagnosis of a benign lesion is safely established, the surgeon may reduce the intussusception by milking it out in a distal to proximal direction[40], allowing for a limited resection. Wang *et al*[41] report that for enteric intussusceptions due to benign lesions, reduction and limited resection resulted in non-recurrence of intussusception. In patients with a risk of a short bowel syndrome due to multiple small intestinal polyps causing intussusception, such as Peutz-Jeghers syndrome, a combined approach with limited intestinal resections and multiple snare polypectomies should be mandatory[42]. Moreover, in patients complicated with postoperative bowel obstruction due to an intussusception, reduction is also recommended, provided that the bowel appears non-ischemic and viable[4].

Finally, several reports have been published regarding the laparoscopic approach of adult intussusception, due to benign and malignant lesions of the small and large bowel[43-53]. Laparoscopy has been used successfully in selected cases, depending on patients' general status and availability of surgeons with sufficient laparoscopic expertise. After establishing the diagnosis of intussusception and the underlying disease laparoscopically, reduction and/or *en bloc* resection can be performed with the same method.

CONCLUSION

Adult bowel intussusception is a rare but challenging condition for the surgeon. Preoperative diagnosis is usually missed or delayed because of nonspecific and often subacute symptoms, without the pathognomonic clinical picture associated with intussusception in children. Abdominal CT is considered as the most sensitive imaging modality in the diagnosis of intussusception and distinguishes the presence or absence of a lead point. Due to the fact that adult intussusception is often frequently associated with malignant organic lesions, surgical intervention is necessary. Treatment usually requires formal resection of the involved bowel segment. Reduction can be attempted in small bowel intussusceptions provided that the segment involved is viable or a malignancy is not suspected.

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REVIEW

Midkine translocated to nucleoli and involved in carcinogenesis

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Abstract

Midkine (MK) is a heparin-binding growth factor with its gene first identified in embryonal carcinoma cells at early stages of retinoic acid-induced differentiation. MK is frequently and highly expressed in a variety of human carcinomas. Furthermore, the blood MK level is frequently elevated with advance of human carcinomas, decreased after surgical removal of the tumors. Thus, it is expected to become a promising marker for evaluating the progress of carcinomas. There is mounting evidence that MK plays a significant role in carcinogenesis-related activities, such as proliferation, migration, anti-apoptosis, mitogenesis, transforming, and angiogenesis. In addition, siRNA and anti-sense oligonucleotides for MK have yielded great effects in anti-tumor activities. Therefore, MK appears to be a potential candidate molecular target of therapy for human carcinomas. In this paper, we review MK targeting at nucleoli in different tumor cells and its role in carcinogenesis to deepen our understanding of the mechanism of MK involved in carcinogenesis.

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Key words: Midkine; Nuclear localization; Carcinogenesis

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INTRODUCTION

Midkine (MK), also known as MDK, FLJ27379, and NEGF2, is a 13-kDa heparin-binding growth factor with a high affinity for heparin, which shares a 50% homology in amino acid sequence with pleiotrophin (PTN), another member of MK family. Previous findings indicate that MK could be internalized depending on lipoprotein receptor-related protein (LRP), a low-density lipoprotein (LDL) receptor-related protein, and is further transported to nuclei depending on nucleolin. In our study, MK could be translocated to and accumulated in nuclei and nucleoli of different kinds of tumor cell[1]. Furthermore, K79R81, K86K87, and C-terminal tail of MK constitute the nuclear localization determinants of MK. The C-terminal tail is the key element controlling MK nucleoli accumulation though the N-terminal tail. K79R81 and K86K87 also contribute to this process. Immunogold-labeling electron microscopy can investigate the exact location of MK mainly localized in granular component (GC), dense fibrillar component (DFC) and the border between DFC and FC[1]. The phenomenon of nuclear targeting of MK is related to the promotion of cell survival and anti-apoptotic activity[2].

MK is detectable in various carcinomas such as breast, prostate, grastric, colon, hepatocellular and urinary bladder carcinomas, neuroblastoma, glioma and Wilms' tumor, exhibiting a proto-oncogene function[3-6]. However, its expression is low or undetectable in adult normal tissues. MK is deeply involved in cancer progression, onset of inflammatory diseases and repair of injured tissues. MK suppresses tumor growth both *in vitro* and *in vivo* and it is believed that MK is a new therapeutic target for curing tumors.

MK TRANSLOCATED TO AND ACCUMULATED IN NUCLEI

It has been reported that MK localizes in the nuclei[2,7], nucleoli[8], or cytosol[9], which is inconsistent with the intracellular localization of MK. Human MK exclusively

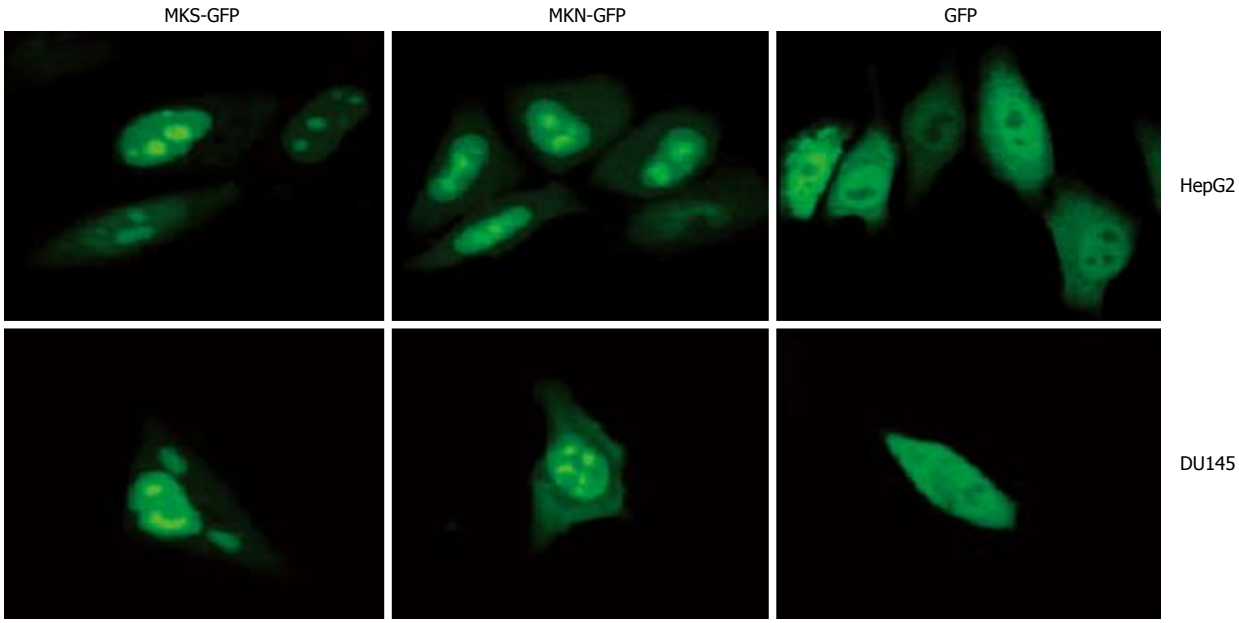

Figure 1 Subcellular localization analysis of MKS-GFP and MKN-GFP fusion proteins in carcinoma cell lines. MKS-GFP and MKN-GFP fusion proteins were localized to nuclei and accumulated in nucleoli, but rarely localized to cytoplasm. The high light "spots" in the center of nuclei are nucleoli. HepG2 cells (upper panel) and DU145 cells (bottom panel) are transfected with pEGFP-N2, and diffuse freely in the whole cell except in nucleoli.

localizes in nuclei and nucleoli in HepG2, DU145, and MCF7 cells by using GFP as a tracking molecule. GFP protein is stable *in vivo* and fused to the C or N terminus of many cellular and extracellular proteins without loss of activity, permitting tagging of proteins for gene regulation analysis, protein localization, or specific organelle labeling, and thus can be used as a perfect positive control to detect the information of MK. We cloned two MK gene fragments with or without signal peptide and separately inserted them into pEGFP-N2 plasmids (enhanced green fluorescent protein N-terminal protein fusion vector). Then, two recombinant plasmids pEGFP-MKS (EGFP fused with MK with signal peptide) and pEGFP-MKN (EGFP fused with MK without signal peptide) were obtained. The results showed that 24 h after transfection, both fusion proteins of MKS-GFP and MKN-GFP in the two cell lines, HepG2 and DU145, were localized exclusively in the nuclei and accumulated in the nucleoli, while GFP control was detected diffusely over the entire cell body except in nucleoli, indicating that signal peptide in pEGFP-MKS does not participate in nuclear and nucleolar localization (Figure 1). Similar results were obtained in MCF7 cell line (data not shown).

MK IS INTERNALIZED IN A LRP-DEPENDENT MANNER AND PROTEASOMAL DEGRADATION

Lipoprotein receptor-related protein (LRP), also known as a low-density lipoprotein (LDL), is a midkine-binding protein[10]. LRP belongs to the LDL receptor family, which includes five prototype members: LDL receptor, ApoE receptor2, very low-density lipoprotein (VLDL) receptor, LRP, and LRP2/megalin. The major functions of these receptors are to endocytose and deliver their ligands to lysosomes for degradation or catabolism[11,12]. MK is not internalized in LRP-deficient cells, whereas transfection of a LRP expression vector can restore MK internalization and subsequent nuclear translocation, suggesting that LRP binds to heparin-binding growth factor, MK, and mediates nuclear targeting by MK. Internalized MK in cytoplasm binds to nucleolin, a nucleocytoplasmic shuttle protein and tanslocates to nuclei. When the nuclear localization signal located next to the acidic stretches is deleted, the mutant nucleolin not only accumulates in cytoplasm but also suppresses the nuclear translocation of MK. With respect to nuclear targeting by MK, 37-kDa laminin-binding protein precursor (LBP) binds to MK and is cotranslocated with MK into nuclei[7]. It is possible that MK uses both nucleolin and LBP precursor as shuttle proteins, revealing a novel role of LRP in intracellular signaling by its ligand, and the importance of nucleolin and LBP in the process of nuclear target of MK (Figure 2).

It is widely held that growth factor signaling is terminated by lysosomal degradation of its activated receptor and endocytosed growth factor is transported to lysosome. Nuclear targeting is another important pathway through which signals of growth factors are mediated. The nuclear targeting pathway is down-regulated by the proteasome system. Degradation of endocytosed MK is suppressed by both proteasome and lysosome inhibitors. By contrast, proteasome inhibitor rather than lysosome inhibitor accelerates the nuclear accumulation of MK. Expression vector of less signal sequence MK in cytosol, can be constructed because endocytosed MK may be translocated to cytosol from cellular compartments before entering nuclei. Cytosol-produced MK undergoes proteasomal degradation, accumulates in nuclei as endocytosed MK, and is polyubiquitinated with its nuclear accumulation increased by proteasome inhibitors. MK

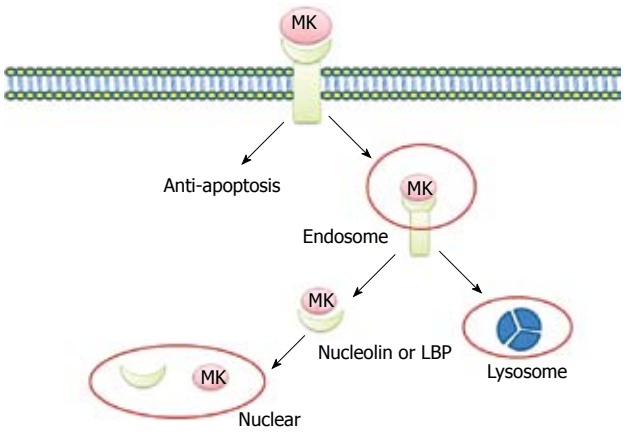

Figure 2 Nuclear targeting by growth factor MK. MK was endocytosed into cell cytosol through binding to the receptor of LRP, and then tanslocated to nuclei in the presence of nucleulin or LBP. At the same time, the internalized MK is degraded by proteasome to "off" the signals from the cell surface receptors.

molecule can be further dissected by cytosol-produced MK to investigate its role in degradation and trafficking. The N terminal half-domain of MK is more significantly susceptible to proteasomal degradation, whereas the C terminal half-domain is sufficient to locate nuclei. These data highlight the desensitization of nuclear targeting by growth factor MK and indicate that the proteasome system plays a critical role in desensitization of nuclear targeting. MK is prone to proteasomal degradation[13]. Because "off" signaling is essential for life, it is reasonable that nuclear targeting growth factor MK is prone to degradation by proteasome (Figure 2).

CONFORMATIONAL DETERMINANTS OF INTRACELLULAR LOCALIZATION OF MK

Nuclei orchestrate the running of cells and is a highly dynamic organelle containing dynamic compartments created by relatively immobile binding or assembly sites[14]. In order to function properly, nuclei should rely on a constant flow of molecules between cytosol and nuclei. Many proteins can be translocated into nuclei by the presence of import signals such as nuclear localization signal or sequence (NLS) which can be specifically recognized by receptors on nuclear pore complex[15], or certain shuttling proteins such as nucleolin[16]. After transported into nuclei, proteins can be docked at different subnuclear compartments. In subnuclear structures such as nucleoli, proteins may accumulate in a steady-state compartment mediated by nucleolar localization sequence (NoLS) or domain which might interact with local high-affinity binding sites[17]. We have identified the conformational determinants for MK nuclear and nucleolar localization[18], showing that K79R81, K86K87, and C-terminal tail of MK constitute the nuclear localization determinant, and play an important role in nuclear localization.

DISTRIBUTION OF MK IN NUCLEOLI

At ultra-structure level, nucleoli include three components: fibrillar center (FC), dense fibrillar component (DFC), and granular component (GC)[19,20]. Although MK tanslocated to nucleoli and accumulated in nucleoli, its exact location in nucleoli is still unclear. Immunogold-labeling electron microscopy shows that MK is mainly localized in GC, DFC and the border between DFC and FC (data unpublished). Because each component is corresponding to a special biological function, the nascent transcripts appear in the junction region between FC and DFC, accumulate in DFC and continue intranucleolar migration of rRNA towards GC[21,22], suggesting that the role of MK in nucleoli may be related to the control of rRNA gene transcription, pre-rRNA processing, and nascent ribosome subunit assembly, the downstream elements to control cell proliferation.

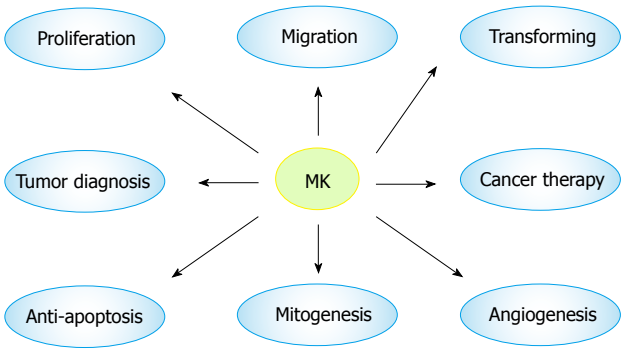

Figure 3 Biological function and medical application of MK. The most attractive feature of MK is its involvement in carcinogenesis, which plays a great role in proliferation, migration, anti-apoptosis, mitogenesis, transformation, and angiogenesis. Furthermore, MK is expected to be a target molecule of diagnosis of and therapy for tumor.

INVOLVEMENT OF MK IN CARCINOGENESIS

MK exhibits various cancer-related activities and is involved in carcinogenesis and cancer advancement. MK exerts its fibrinolytic activity by enhancing the level of plasminogen activator in endothelial cells[23], and transforms NIH3T3 cells[24]. MK promotes migration of neutrophils, osteoblastic, osteosarcoma cells, neural cells, and macrophages, which is always dependent on the receptors: N-syndecan and PTPζ[25]. MK mediates cell survival and growth mainly through P13-kinase and extracellular signal-regulated kinase (ERK) in intracellular signaling[26]. MK exerts its anti-apoptotic activity to rescue Wilms' tumor cells from cisplatin[27]. MK also induces a strong angiogenic response in rabbit cornea, when its cDNA is transfected[28]. Resistance to cytotoxic agents is a major limitation for its use in treatment of human cancers. MK has been recently identified as a mediator of intercellular transfer of drug resistance[29] (Figure 3).

MEDICAL APPLICATION OFMKIN CANCER THERAPY

MK is frequently over-expressed in most carcinomas[30].

Serum and urinary MK levels are becoming prognostic and diagnostic markers of various tumors. Furthermore, MK demonstrates many activities that are significantly correlated to carcinogenesis, indicating that MK is a candidate target of therapy for carcinomas.

MOLECULAR TARGET

Anti-sense ODNs have a huge potential as agents to turn off the expression of specific proteins *in vitro* and *in vivo*. Indeed, anti-sense MK ODNs show anti-tumor activities in various carcinoma cells. Moreover, they also suppress the growth of pregrown tumors in nude mice via atelocollagen-mediated gene transfer, and exert their inhibitory effect on mitosis of cancer cells[31-33]. Thus, abolition of MK production or disruption of its signaling pathway is a good treatment modality for human carcinomas.

GENE TARGET

Currently, studies on gene therapy for cancer are carried out. The most difficult aspect of developing an *in vivo* approach to cancer is correctly targeting cancer cells. Many vectors target tumors for gene delivery, including viral and synthetic vectors (liposome and emulsion), which show promise in targeting cancer cells. It is essential to use a strong and tissue-specific promoter region if a suicide gene is to be expressed selectively in cancer cells. Compared with cytomegalovirus (CMV) promoter, MK promoter exhibits a stronger expression in Wilms' tumor cells than CMV promoter[34]. Furthermore, adenovirus containing CMV promoter-thymidine kinase gene can cause severe side effect to liver, while the MK promoter-thymidine kinase gene does not have such a side effect. Therefore, a suicide gene under the control of MK promoter is a highly potential strategy for the treatment of carcinomas[35].

CONCLUSION AND PERSPECTIVES

In this review, we have described that MK can be translocated to nucleoli, where it accumulates in different tumor cells. A growing body of evidence indicates that nuclear targeting plays an indispensable role in the biological activities of growth factors. For example, nuclear localization of fibroblast growth factor 1 and Schwannoma-derived growth factor is necessary for their mitogenic activity[36,37]. Increased ribosomal gene transcription and cell proliferation are closely correlated to the nuclear translocation of fibroblast growth factor-2[38]. It is, thus, important to make clear that signals from tumor cell surface receptors and ligands translocated to nuclei play a role in the biological activities of MK.

MK is comparatively ubiquitous in many kinds of tumor, whereas its expression is usually low or undetectable in normal adult tissue. Serum MK levels are also elevated in patients with various carcinomas, such as gastric, colon, hepatocellular, and lung carcinomas[27]. These observations indicate that MK should be considered indispensable to the development and enlargement of tumors. Further analyses of MK mechanisms related to carcinogenesis should shed light on therapies for tumors and cancers. To further establish blood MK as a tumor marker, prospective studies on blood levels and prognosis will provide useful information. Further study should focus on the application of MK in gene therapy for cancer.

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TOPIC HIGHLIGHT

Juan-Ramón Larrubia, PhD, *Series Editor*

Mycobacterium avium subspecies *paratuberculosis* and its relationship with Crohn's disease

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Abstract

The hypothesis postulating that *Mycobacterium avium paratuberculosis* (MAP) is the cause of Crohn's disease (CD) has been circulating for many years. Advances in molecular techniques, such as polymerase chain reaction and culture methods, have enabled researchers to demonstrate that there is an association between MAP and CD. Recently, genome-wide association studies have identified novel susceptibility genes for CD, which are critical for generation of an adaptive immune response that is protective against intracellular pathogens, including *M. tuberculosis* infection. However, the role of MAP as a cause of CD suffered a setback with the report that administration of antimycobacterial therapy failed to lead to a sustained response in CD patients. Accordingly, this review sought neither to confirm nor refute this, but instead to survey recent literature on the role of MAP in CD.

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Key words: *Mycobacterium avium* subspecies *paratuberculosis*; Crohn's diasease; Inflammatory bowel disease

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are two related, chronic, remitting and relapsing inflammatory diseases of the gastrointestinal tract, commonly known as inflammatory bowel diseases (IBDs). While the causes of IBDs are unknown, it is thought that inflammation results from an inappropriate chronic activation of the innate and adaptive mucosal immune systems in a genetically susceptible host, and that enteric microflorae play a pivotal role in the initiation and maintenance of disease[1]. The key factors responsible for IBDs are fairly well defined, namely, the environment, genetic makeup, commensal florae, and immune response. Insofar as the components of IBD pathogenesis are concerned, investigation of the role of infectious agents and gut commensal florae is an area in which relatively less progress has been made[2]. One of the most controversial questions is whether or not any given microbe plays a role in promoting disease. The similarities between CD and some forms of infectious enterocolitis are sufficiently evident for numerous specific microbial etiologies for CD to have been proposed over the years, including *Pseudomonas maltophilia*, *Mycobacterium kansasii*, *Chlamydia trachomatis*, *Bacteroides fragilis*, *Listeria monocytogenes, Escherichia coli* and *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Just recently, the status of MAP as an infectious cause of CD-an enduring and controversial topic-suffered a severe setback in the form of a multicenter study which reported that administration of antimycobacterial therapy over 2 years afforded no substantial or prolonged benefits for patients with CD[3]. However, MAP is a recurrent candidate for several reasons: it causes epidemic chronic colitis in cattle and other species, including primates; it is reportedly detectable in the intestinal tissues and blood of many CD patients; antibodies to the organism are often disease-associated; and in some cases,

antimycobacterial drugs ameliorate disease.

Four not necessarily mutually exclusive mechanisms have been proposed to drive pathogenic immunologic responses to luminal microbial antigens: (1) microbial pathogens induce intestinal inflammation, (2) dysbiosis of commensal microbiota, (3) host genetic defects in containing commensal microbiota, (4) defective host immunoregulation. These mechanisms increase exposure of bacterial antigens to mucosal T cells or alter host immune responses to commensal bacteria. The detection of MAP DNA in the blood of CD patients may suggest that this organism is a persistent pathogen in CD. However, these results could be a secondary phenomenon due to increased gut permeability or the inability of macrophages in CD to kill MAP. The increased gut leakiness hypothesis is supported by the more frequent detection of other organisms in CD patients compared with controls and suggests a lack of specificity of MAP detection.

Another recent study supports the possibility that *M. paratuberculosis*, though not itself a very effective pathogen in humans, might induce local suppression of phagocyte function in infected tissue, and that this, in turn, might lead to chronic replication within macrophages of other bacteria, including perhaps the mucosal *E. coli* isolates that have now been found in CD tissue samples by several independent groups[4].

MAP

MAP is a member of the *M. avium* complex. *M. avium* strains are widely distributed in the environment and also inhabit normal animal and human intestines. *M. avium* strains do not usually cause disease unless the host is debilitated or immunocompromised. MAP, in contrast, is a specific pathogen with the ability to cause chronic inflammation of the intestine (Johne's disease) in many species, including ruminants and primates[5]. Moreover, it has been suggested that MAP can exist in animal tissue for years without causing clinical disease. Initial clinical signs follow a prolonged incubation period of 2 to 10 years, depending upon the exposure level and the individual animal's ability to fight the infection[6]. MAP invades macrophages in lymphoid tissue in the ileum, where it inhibits phagosome maturation and induces the recruitment of inflammatory cells, resulting in granulomatous enteritis. Characteristics distinguishing MAP from other *Mycobacterium* spp. include: its extremely slow growth; its inability to produce mycobactin; its possession of the insertion element IS900 that occurs as 14-18 copies within the *MAP* genome[7]. The DNA sequence, IS900, is considered the "gold standard" for differentiating MAP from other mycobacteria[8]. Although other IS900-like elements have been described in environmental mycobacteria, the entire IS900 gene is unique to MAP[9].

In common with other mycobacteria, MAP possesses a thick, waxy cell wall containing 60% lipid, which confers on it the properties of acid fastness (the ability to resist decolorization by acidified alcohol), hydrophobicity, and increased resistance to chemicals (e.g. chlorine) and physical processes (e.g. pasteurization)[7]. At least two potential modes of MAP transmission from the host animal to humans have been hypothesized, i.e. ingestion of either contaminated water or milk[10]. The detection of cell wall-deficient forms, also known as spheroplasts, in tissue cultures from affected humans, and subsequent identification of the agent as positive for the IS900 insertion sequence found in MAP by polymerase chain reaction (PCR) and *in situ* hybridization (ISH), has been the basis for suggesting MAP as a potential cause of CD[11]. Hence, MAP spheroplasts may play a role in the development of these human diseases, as well as in paucibacillary forms of Johne's disease in other species.

DETECTION OF MAP IN CROHN'S DISEASE

The association of MAP with CD is supported by identification of MAP in CD patients but not in appropriate controls. The gold standard for detection of MAP is based on isolation of the organism through culture methods. However, such methods are time consuming, because of the organism's fastidious nature and slow growth. Molecular and serologic methods are widely-used alternatives[12].

MAP CULTURE

Advances in culture methods now enable researchers to grow MAP from intestinal tissue, milk, and blood in CD patients[13]. The introduction of commercially available BACTEC and MGIT liquid culture systems, together with the application of IS900 PCR to such cultures, have led to substantial improvements in the ability to detect subclinical MAP infection in ruminants[14]. Some researchers have suggested that bacterial culture using liquid media has greater analytical sensitivity than that using solid media. Recently, two types of culture media have been shown to determine differential growth of MAP strains. This should be borne in mind when evaluating the detection capabilities of diagnostic tests or interpreting data from molecular epidemiologic studies performed using different type of culture medium[15]. MAP has been cultured from the milk of two women with active CD, who were breastfeeding[16]. This observation has not been replicated by other researchers. In another study[17], MAP was recently cultured in 50% of blood samples from CD patients, 20% of samples from UC patients and 0% of samples from healthy controls. In this study, the observation that MAP could be cultured from CD patients did not correlate with the use of immunosuppressive medications. Finally, MAP has also been cultured in a higher percentage of bowel-pinch biopsies from CD patients (42%) than from controls (9%)[18]. A preliminary report issued by a National Institutes of Health-sponsored blinded study

showed no differences in the culture recovery rates of two independent laboratories, and no detection of MAP 16s rRNA[19].

DETECTION OF THE INSERTION SEQUENCE IS900

The two main methods used to detect the insertion sequence IS900 include PCR and ISH assays. Previous studies have shown that reliable and reproducible detection of *MAP* by PCR applied directly to DNA extracted from human tissue and other samples, is extraordinarily difficult. The use of suboptimal sample-processing procedures results in false-negative results[18]. The results of MAP detection using nucleic acid-based techniques have recently been reported in two meta-analyses. These suggest that there is sufficient evidence for the presence of MAP in the gut of CD patients, regardless of whether CD patients are compared with individuals without inflammatory bowel disease or with UC patients[9,12]. Nevertheless, this association remains controversial and inconclusive. PCR data are open to criticism because the technique assays DNA that could come, either from live bacteria, or merely from the scattered debris of killer organisms and thus be of questionable biologic importance[13].

SEROLOGIC STUDIES OF MAP

Another approach to studying the possible role of MAP in the etiology of CD is to evaluate CD patients for the presence of antibodies reactive against MAP antigens[20]. Serologic tests for diagnosis of paratuberculosis, such as agar gel immunodiffusion, enzyme-linked immunosorbent assay and complement-fixation, are relatively easy to perform but suffer from a lack of sensitivity[10]. In a recent meta-analysis[12], the prevalence of MAP antibodies was higher in CD patients than in controls in most of the studies but there were high levels of inter-study heterogeneity. For the studies using serologic markers, the sources of heterogeneity remained unclear: confounding factors, bias and differences in study populations are all likely to have contributed to heterogeneity[12]. The p35 and p36 antigens were the most frequently used, and several studies have shown that CD patients display specific reactivity to the p35 and p36 antigens[21]. However, both p35 and p36 are present in *M. avium* subsp. *avium*, suggesting that a similar reaction would be obtained with the *M. avium* subsp. *avium* p35 and p36 homologs[20]. Accordingly, though positive tests were more common among CD patients than among UC patients and controls, this is not necessarily because of MAP infection but may be attributable to other MAP-like bacteria.

MAP AND GENETIC SUSCEPTIBILITY TO CROHN'S DISEASE

The simultaneous discovery, by two groups using positional cloning and candidate-gene approaches, of Nucleotide Oligomerization Domain 2/Caspase Recruitment Domain 15 (NOD2/CARD15*)* as a susceptibility gene for CD[22], provided specific support for the long-held theory that a genetically dysregulated host immune response to luminal bacteria results in CD. Muramyl dipeptide, a component of bacterial peptidoglycan, is recognized by the NOD2 receptor; nonetheless, the exact mechanism whereby NOD2 polymorphisms contribute to increased propensity to develop CD is still not completely understood. NOD2/CARD15 deficiency induces abnormal development and function of Peyer's patches, characterized by an exaggerated immune response and increased permeability[23]. Thus, patients carrying CARD15/NOD2 mutations are unable to control bacterial infections, which results in an inadequate innate response to bacterial invasion and enables bacteria to accumulate[24]. CARD15/NOD2 mutations are associated with defective clearance of invasive *Salmonella* infection in epithelial cells. Hence, an attractive explanation linking CARD15/*NOD2* to CD is that of ineffective clearance of intracellular MAP infection: indeed, a recent clinical study has shown that the mononuclear cells of CD patients, which are mutant for NOD2, display defective recognition of MAP bacteria[25].

However, no association between MAP serologies and NOD2 polymorphisms was observed in a large population-based study conducted in Manitoba[26]. Elsewhere, in a small-scale Sardinian study[27], the possibility of an interaction between MAP positivity and NOD-2 gene mutations was raised, with the authors suggesting that there might be a trend towards an association between the presence of CARD15/NOD2 mutations and MAP-positive status. However, this association was only present when CD subjects were compared against controls who had CARD15/NOD2 mutations, whereas having NOD2 mutations had no impact on MAP status among the CD subjects[27]. The same team failed to observe any association between SLC11A1 polymorphisms in the Sardinian population and MAP infection[28].

A genome-wide association study identified the interleukin-23 receptor (IL-23R) as a novel susceptibility gene for CD[29]. Mounting evidence suggests that IL-23, which is similar to IL-12, is critical for generation of an adaptive immune response that is protective vis-à-vis intracellular pathogens, including *M. tuberculosis* infection[30]. Recently, two studies demonstrated an association between CD and a coding variant of autophagy-related-16-like 1 (ATG16L) gene[31] and IRGM gene[32], thereby implicating the autophagy pathway of the innate immune system. The autophagy trafficking pathway is critical in inhibiting *M. tuberculosis* survival in infected macrophages[33]. Accordingly, infection of a subset of CD patients with intracellular killing defects caused by ATG16L1, IRGM, IL-23, NCF4, or any other as yet unreported gene, needs to be investigated[34].

ANTIMYCOBACTERIAL ANTIBIOTICS FOR CROHN'S DISEASE

Another approach to identifying disease causation is the use of chemotherapeutic agents to eliminate the infectious agents. The most irrefutable evidence of the fact that a microbial agent causes a given disease is long-term remission of clinical manifestations and an altered natural history of disease following clearance of the infection. In common with other atypical mycobacteria, MAP has characteristics that limit the number and type of potentially effective antibiotics[35]. *In vitro* sensitivity analyses show that clinical isolates of MAP are not responsive to traditional anti-*M. tuberculosis* agents, and so isoniazid, ethambutol, and rifampicin are not effective. Clarithromycin and azithromycin are considered to be the most effective drugs for treatment of MAP. In 2000, a meta-analysis[36] suggested that antimycobacterial treatment may be effective in maintaining remission achieved by corticosteroids. Treatment of CD with antimycobacterial therapy does not seem to be effective without a course of corticosteroids to induce remission. However, because of the small number of studies included in this meta-analysis and the heterogeneity of the trials, which used a wide range of antibiotic combinations administered for variable periods, these results should be interpreted with caution. Lastly, the largest study, a well-designed, randomized, placebo-controlled trial of clarithromycin, rifabutin and ethambutol, failed to show a sustained response in CD patients. Although the antibiotics registered a short-term benefit at 16 wk, in addition to the effect of the corticosteroid therapy, the study showed no prolonged advantage of the antibiotic combination, whether during the 2-year treatment phase or, more importantly, after the therapy had been halted[3].

An argument against a role for MAP in CD is that, if CD were indeed a chronic mycobacterial infection, then immunosuppressive therapies (corticosteroids, thiopurine drugs and tumor necrosis factor (TNF)-α suppressive therapies) should be associated, not with improvement, but rather with increased rates and severity of mycobacterial disease[35]. Currently, there is no published evidence, clinical or experimental, to establish whether or not MAP infection is exacerbated by TNF-α antibodies[37]. In one study[38], corticosteroid therapy was associated with lower MAP detection rates. However, in the case of the most commonly used immunosuppressive drugs used to treat CD symptoms, such as thiopurine drugs, e.g. azathioprine, and their metabolites, e.g. 6-mercaptopurine, inhibit the growth of MAP *in vitro*[39]. It is possible that intracellular cell deficient (spheroplast) MAP may not replicate well despite immunosuppression[35]. A recent study[40] showed that antimycobacterial and thiopurine drugs used in concert may produce an interactive effect. The apparent bacteriostatic effects of 6-mercaptopurine on *M. paratuberculosis* rendered the organism less susceptible to the bactericidal effects of antibiotics. These findings should also influence the design of therapeutic trials aimed at evaluating antibiotic treatments of CD: thiopurine drugs may confound interpretation of data on therapeutic responses for both antibiotic-treated and control groups.

EPIDEMIOLOGIC EVIDENCE FOR MAP AS A CAUSE OF CD

Other arguments against a role for MAP in CD are based on the difficulty of reconciling some of the epidemiologic features of CD with a causative role for chronic MAP infection[41]. First, farmers (and persons in rural settings) should be at increased risk of a livestock-associated pathogen, yet there is no evidence that they have increased rates of CD[42]. Second, environmental conditions, such as poor sanitation and overcrowding which should favor transmission of an infection, actually appear to protect against CD. Third, there is a remarkable paucity of evidence for vertical or horizontal transmission of CD[41]. Finally, detection of MAP in CD is neither disease- nor bacterium-specific. Detection of bacterial DNA in the granulomas of intestinal CD is not specific to MAP, in that other forms of bacterial DNA are also present[43]. However, a study which reviewed epidemiologic models of disease causation, has concluded that the current epidemiologic evidence strongly supports the conjecture (especially among those who believe in the theory of inductivism) that CD is caused by MAP[5]. Inductivism is one of the major philosophical doctrines about causation. This doctrine holds that science proceeds from observation to theory, beginning with observations derived from experiments, and extrapolating from these to general laws[5].

CONCLUSION

MAP is the causative agent of Johne's disease. It seems likely that chronic infection with MAP does occasionally occur in humans. MAP is widely present in our food chain and the DNA of this organism can be recovered from the intestine of CD patients. Studies have shown that a high percentage of subjects with CD are infected with MAP, though whether the association of this bacterium and CD is causal or coincidental is not known. Epidemiologists have gathered enough information to indicate an association between MAP and CD. Nonetheless, the role of MAP in CD etiology is not known, and may be determined from consistent results of studies using improved methods of isolation and detection of MAP bacilli and/or MAP-elicited immune responses in the host.

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TOPIC HIGHLIGHT

Juan-Ramón Larrubia, PhD, *Series Editor*

DNA-guided hepatitis B treatment, viral load is essential, but not sufficient

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Abstract

Hepatitis B virus (HBV) infection is a global public health problem that concerns 350 million people worldwide. Individuals with chronic hepatitis B (CHB) are at increased risk of developing liver cirrhosis, hepatic de-compensation and hepatocellular carcinoma. To maintain undetectable viral load reduces chronic infection complications. There is no treatment that eradicates HBV infection. Current drugs are expensive, are associated with adverse events, and are of limited efficacy. Current guidelines try to standardize the clinical practice. Nevertheless, controversy remains about management of asymptomatic patients with CHB who are hepatitis B e antigen (HBeAg)-positive with normal alanine aminotransferase, and what is the cut-off value of viral load to distinguish HBeAg-negative CHB patients and inactive carriers. We discuss in detail why DNA level alone is not sufficient to begin treatment of CHB.

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Key words: Hepatitis B virus; Viral DNA; Alanine transaminase; Antiviral drug; Hepatitis B e antigen; Antiviral drug resistance

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health problem. An estimated 350 million people worldwide are chronically infected with HBV. Approximately 500 000 die annually from HBV-related liver disease[1]. The prevalence and concerns to public health institutions about HBV infection vary according to geographical origin.

Individuals with chronic hepatitis B (CHB) are at increased risk of developing serious problems including liver cirrhosis, hepatic de-compensation and hepatocellular carcinoma (HCC). Fifteen to forty percent of these individuals will develop serious sequelae during their lifetime and have greater evolution to cirrhosis or HCC[2,3]. The 5-year rate of progression from CHB to cirrhosis is estimated to be 12%-20%[4-7]. In patients with cirrhosis, the 5-year cumulative risk of developing HCC is 17% in East Asia and 10% in Western Europe and the United States, and the 5-year liver-related death rate is 15% in Europe and 14% in East Asia[8,9]. Seropositivity for the hepatitis B surface antigen (HBsAg) is one of the most important risk factors for HCC[10]. Seropositivity for hepatitis B e antigen (HBeAg) is associated with an increased risk for HCC, and it is significant regardless of serum level of alanine aminotransferase (ALT) and status of liver cirrhosis[8,10,11]. The risk of progression appears to be greatest in patients who progress from an immunotolerant to an immune-clearance phase[12], in patients who have delayed HBeAg seroconversion[13], and in patients who have reactivation of HBV replication after HBeAg seroconversion[14-16].

Disease progression is variable and multifactorial. It is influenced by several factors including replicating activity of the virus, and host and environmental factors[17]. Four phases of CHB have been defined: immunotolerant phase, immune active phase, HBeAg seroconversion to anti-HBeAg, and inactive carrier.

HBV eradication should be useful for patients and society at large. General actions have been developed to prevent HBV infection: universal vaccination at birth, pregnancy screening measures, sexual education, and preventive and prophylactic management of clinical devices. Immigration, cheap air travel and globalization, are changing epidemiological patterns and pre-core mutation prevalence[18]. HBV suppression and reduction of necro-inflammatory activity in CHB may prevent cirrhosis, liver failure and HCC[19,20]. Unfortunately, because of extra-hepatic reservoirs, integration of HBV DNA into the host genome and protected intracellular, covalently closed circular DNA, HBV cannot be eradicated with current therapies. The next best alternative is sustained suppression of HBV replication until HBV DNA is undetectable. A viral load of > 10000 copies/mL (2000 IU/mL) is a strong risk predictor of HCC, independent of HBeAg status, ALT level and liver cirrhosis[10,21,22]. Secondary targets are ALT normalization (biochemical response), hepatic necro-inflammatory improvement (histological response) and HBeAg seroconversion. Complete response includes fulfilling biochemical criteria, virological response and loss of HBsAg.

CONSENSUS GUIDELINES

There are consensus guidelines that help the clinician to make decisions about whether or not to treat a patient[23-27]. Treatment decision items are: HBeAg-positive or HBeAg-negative, serum viral load measured by polymerase chain reaction, elevated transaminases, histological lesions, duration of CHB (age > 40 years is likely to be a surrogate marker of disease duration) and family history of HCC. However, there are still areas of controversy in actual treatment of CHB. The long-term efficacy and safety are unknown. The question is who should be treated, how and especially when. The main guidelines are from the American Association for the Study of Liver Disease (AASLD Practice Guidelines)[24]. Their recommendations are summarized.

AASLD practice guidelines (Ⅰ)

Treatment of patients with HBeAg-positive CHB: (1) Treatment should be considered if ALT is > 2 ULN or moderate/severe hepatitis is found upon biopsy, and HBV DNA is > 20000 IU/mL; liver biopsy may be considered prior to treatment if illness is compensated. Treatment should be delayed for 3-6 mo in persons with compensated liver disease, to determine if spontaneous HBeAg seroconversion occurs. Patients with icteric flares or clinical de-compensation should be promptly treated. (2) If ALT is persistently normal or minimally elevated (< 2 ULN) and viral DNA is > 20000 IU/mL, current treatment has low efficacy. Generally, treatment should not be initiated in these patients. Liver biopsy may be considered in patients with fluctuating or minimally elevated ALT, especially in those aged > 40 years old, or those with a family history of HCC. Treatment may be initiated if there is a moderate or severe necro-inflammation or significant fibrosis upon liver biopsy.

Recommendation for monitoring HBeAg-positive patients: (1) Patients with persistently normal ALT should be tested for ALT at 3-6-mo intervals, and for ALT/HBV DNA more often if ALT becomes elevated. (2) HBeAg status should be checked every 6-12 mo. (3) Patients who remain HBeAg-positive with HBV DNA > 20000 IU/mL after 3-6 mo with elevated ALT levels of 1-2 ULN, or DNA levels > 20000 IU/mL and aged < 40 years old, should be considered for liver biopsy. Treatment should be considered if liver biopsy shows moderate/severe inflammation.

AASLD practice guidelines (Ⅱ)

Treatment of patients with HBeAg–negative CHB: (1) If ALT is > 2 × ULN, and HBV DNA is > 2000 IU/mL, treatment should be considered. (2) If ALT is 1-2 × ULN and DNA is 2000-20000 IU/mL, liver biopsy may be considered. Treatment may be initiated if liver biopsy shows moderate/severe necro-inflammation or significant fibrosis. (3) If ALT level is normal and DNA is < 2000 IU/mL, patients should not be treated. Patients should be tested for ALT every 3 mo during the first year to verify that they are truly in the inactive carrier state, and then every 6-12 mo. Test DNA and more frequent monitoring should be performed if ALT or AST increases above the normal limit. Patients should be observed and treated if HBV DNA or ALT increase.

HbeAg-negative and anti-HBeAg-positive patients with cirrhosis: Patients with compensated cirrhosis and HBV DNA > 2000 IU/mL and those with de-compensated cirrhosis and detectable HBV DNA by PCR assay should be considered for antiviral therapy, regardless of serum ALT level. The aim of the treatment in CHB patients is to achieve a maximum decrease in viral load. If serum DNA levels decrease to undetectable levels, by PCR, there is a reduction, not absence, of complications associated with HBV.

AASLD PRACTICE GUIDELINES

The new AASLD practice guidelines follow the traditional criteria of treatment, but, as with other guidelines[26,27], incorporate current tendencies to increase the viral load value. This adds the recommendation of treatment for HBeAg-negative patients with serum DNA level > 2000 IU/mL (10^4 copies/mL) and ALT < 2 × ULN, if biopsy shows moderate/severe necro-inflammation and significant fibrosis.

There is no doubt in the management of cases according to the guidelines. There are two areas of controversy. Management of asymptomatic HBeAg-positive patients with normal ALT levels and without the following criteria: advanced histological findings, recurrent hepatitis flares, age > 40 years with persistently high HBV DNA levels, and family history of HCC. The other area of ongoing debate is the differentiation between the inactive carrier state and asymptomatic

HbeAg-negative CHB with normal ALT. We do not know what is the cut-off value of DNA used to define these two phases of CHB infection. The main question in CHB treatment is if the new antiviral therapies permit treatment only guided by DNA levels and ignore other factors such as ALT level or histology.

Lok and McMahon consider that treatment is indicated if the risk of liver–related mortality and morbidity in the near future (5-10 years), and the likelihood of achieving maintained viral suppression after a defined course of treatment are high[24]. However, present therapies have a high cost and limited efficacy. Response to treatment is limited by adverse events [interferon (IFN)α and pegylated (PEG)-IFNα], drug interactions, drug resistance [nucleotide and nucleoside analogs (NAs)], relapses after suspension of treatment, and limited adherence to long-term treatments.

It may be that the approach to treatment of CHB patients with normal ALT must be done on an individual basis, and must consider serum viral levels measured by PCR, elevated transaminases, histological lesions, age, CHB phases, and family history of HCC. The response to treatment changes with the different phases of illness.

VIRAL LOAD AND TREATMENT: FAVORABLE ARGUMENTS

It is known that maintained high levels of HBV DNA are associated with progressive liver disease. Serum DNA levels are a prognostic factor, and contribute to define the phase of CHB infection, the treatment indication, and allow an assessment of the efficacy of antiviral therapy. High levels of HBV DNA are an independent risk factor for cirrhosis[28] and HCC in Asia[22,29].

The REVEAL (Risk Evaluation of Viraemia Elevation and Associated Liver Disease)-HBV study group followed a cohort of 3653 HBsAg-positive patients in Taiwan. Their average age was 45 years and they acquired HBV infection perinatally. A baseline high HBV-DNA level > 10000 copies/mL was associated with a significant increased risk of HCC[30] and with progression towards cirrhosis[22]. Those patients that have persistently high levels of viral replication, for up to four decades, are at highest risk for HCC/cirrhosis after adjusting for HbeAg status, age, sex, ALT level, cigarette smoking and alcohol consumption. Many Asian patients may be in an immunotolerant phase, and the applicability of these data to western countries is not clear.

However, Fattovich *et al*[28] have re-evaluated a cohort of 70 Caucasian patients with HBeAg-positive chronic hepatitis at presentation, and they have shown that the risk of liver-related mortality in Caucasian adults with chronic hepatitis is strongly related to sustained disease activity and ongoing high levels of HBV replication, irrespective of HBeAg status. Other risk factors for liver-related death are older age, male sex, cirrhosis at entry, and absence of sustained remission.

It seems that a high level of HBV DNA is an independent risk factor for development of cirrhosis and HCC. The correlation between HBV DNA level and degree of liver injury upon biopsy is not well characterized. Serum HBV DNA level of > 100000 copies/mL is not correlated with histological grade or stage of liver disease in CHB patients, whatever the status of HBeAg[9]. Kumar *et al*[31], in a recent, large prospective study, have shown clearly that baseline ALT and DNA level are good predictors of histologically significant fibrosis. However, in an analysis of clinical trial data using NAs, Mommeja-Marin *et al*[32] have shown that there is a strong linear correlation between log-reduction of HBV-DNA levels and improvement in inflammation and fibrosis in liver biopsy.

The interpretation of serum DNA level is not easy, because we do not know the cut-off value for defining indication for and response to treatment. The 2000 National Institutes of Health conference chose an arbitrary value of 20000 UI/mL (> 10^5 copies/mL)[33]. As DNA levels have a fluctuating nature, monitoring changes in serum DNA levels is an essential tool.

VIRAL LOAD AND TREATMENT: UNFAVORABLE ARGUMENTS

Presently, the viral load cannot be considered as the only treatment criterion. HBV DNA persists even in persons who have serological recovery from acute HBV infection[34]. Patients with low HBV-DNA levels, between 300 and 10^4 copies/mL, have, although a very low one, a risk of progression to cirrhosis and HCC[9]. The progression in CHB infection is a multi-factorial process including interaction between host and environmental factors.

ALT value

Host immune response against HBV is essential to control infection. Immune response produces necrosis and inflammation of the liver. Liver biopsy assesses necro-inflammatory activity and cirrhosis, and it is measured indirectly by aminotransferase level. There is no significant interaction between ALT and HBV-DNA levels[35].

Many studies have shown a low prevalence of significant liver injury in CHB patients with normal ALT levels[36-39]. There is a possible bias for including blood donors, and a high proportion of patients in the immunotolerant phase. However, ALT values may vary with body mass, sex, abnormal lipid and carbohydrate metabolism. In addition, recent studies have suggested that ALT is an imperfect marker for liver disease activity. Some studies have detected significant liver injury in CHB patients with normal ALT[40,41]. In a retrospective study, Lai et al have investigated 192 CHB patients, with viral DNA at 10000 copies/mL and hepatic biopsy or clinical cirrhosis. There were significant fibrosis and inflammation in 37% of patients with persistently normal ALT, and a trend for the normal ALT group to include younger patients[42].

Kumar *et al*[31] have found a correlation between

histologically significant fibrosis (F score > 2) and persistently or intermittently elevated baseline ALT and baseline DNA > 10000 copies/mL. However, a normal ALT level in an individual patient does not always indicate absence of significant liver disease. Among HBeAg-positive patients with persistently normal ALT, 60.3% have baseline DNA levels > 5-log copies/mL, 63% have histological activity of hepatitis (HAI) > 3, and 39.7% have fibrosis stage > 2. Concerning HbeAg-negative CHB patients with persistently normal ALT (PNALT), they observed that 35.3% of patients have baseline DNA levels > 5-log copies/mL, 39.7% have HAI > 3, and 13.8% have fibrosis stage > 2. Following ASSLD practical guidelines, the inactive carrier state is differentiated from HBeAg-negative CHB by serial testing of ALT and serum HBV levels, because of the fluctuating pattern of AST/ALT. The inactive carrier state is defined by normal ALT and HBV DNA < 2000 IU/mL (10000 copies/mL). Some studies have observed that a cut-off of 10000 copies/mL may lead to misclassification of 13%-20% of HBeAg-negative CHB patients[43,44]. Kumar *et al*[31] have obtained similar results: 21 of HBeAg-negative patients with baseline DNA levels < 5-log copies/mL and PNALT, have HAI > 3 and fibrosis stage > 2.

Revision of normal limits for ALT level is advisable. The current standards for normal ALT level have been defined by using populations that include persons with subclinical liver disease, chronic HCV infection or non-alcoholic fatty liver disease. We considered normal ALT to be 40 IU/mL and normal aspartate aminotransferase (AST) to be 30 IU/mL. Recently, new upper limits of normal for ALT for men (30 IU/mL) and women (19 IU/mL) have been proposed. Revision of normal limits for ALT level is advisable to obtain a new cut-off, i.e. ALT = 39 IU/mL (men) and 19 IU/mL (women)[45]. Patients with a persistently normal ALT, HBV-DNA level >10000 copies/mL, and significant fibrosis and inflammation upon liver biopsy usually have an ALT in the high range of normal (26-40 IU/mL), and are older than 40 years old. This is consistent with new AASLD guidelines and recent data from Lin *et al*[46], which correlate parameters of progressive disease with high normal ALT. AASLD guidelines suggest decreasing the upper limits of normal for ALT.

ALT, likely with a new-updated cut-off value, is a factor to consider in the treatment of HBC patients. A high ALT value is a predictor of necro-inflammatory activity and a marker of response to actual treatment. In HBeAg-negative patients, its role as predictor of response to therapy is unclear. However, a high ALT level helps to distinguish between the inactive carrier state and asymptomatic HBeAg-negative CHB patients with normal ALT. A normal serum ALT level alone in patients with active viral replication does not predict mild or normal histological findings.

Value of biopsy: fibrosis and necro-inflammatory activity

Patients with moderate/severe inflammation or bridging fibrosis/cirrhosis must be treated[24]. The degree of fibrosis or inflammation upon liver biopsy cannot be predicted for HBV-DNA levels >10000 copies/mL. ALT is an imperfect marker for liver disease (see above). Traditional and current guidelines recommend liver biopsy for patients who meet the criteria for chronic hepatitis (HBsAg positive for > 6 mo, serum HBV DNA > 10^5 copies/mL or > 20000 IU/mL, persistent or intermittent elevation in ALT/AST levels). These patients must be treated if we follow the guidelines, and we believe that liver biopsy may be unnecessary.

Liver biopsy is more important for patients who do not meet the current criteria for treatment but have serum HBV-DNA levels of 10^4 to 10^5 copies/mL (2000-20000 IU/mL) and/or ALT/AST levels that are normal or mildly elevated (< 2 × ULN). The presence of significant inflammation or bridging fibrosis/cirrhosis is an indication for treatment. In a subgroup of these patients, hepatic elastography can avoid the need to carry out a liver biopsy for detection of significant fibrosis. This is a novel non-invasive method to assess hepatic fibrosis in patients with a chronic disease, by measuring liver stiffness. Its failure rate is about 5% of cases, mainly in obese patients[47,48]. Elastography has a high positive predictive value (92%) for diagnosing significant fibrosis (F3 and F4) in Asian CHB patients[49]. This method does not have a defined role in HBeAg-negative patients because histological inflammation during reactivation may affect the results[50].

GENOTYPE VALUE

The relationship between genotype and treatment indication is not well known as yet. The characteristics of the virus during evolution of the illness and the response to the treatment are acquiring an ever-increasingly important role. The prevalence of HBV genotypes varies depending on geographical location. It differs between Asia and Europe, and within European countries. Genotypes B and C are almost the only ones present in Asia. Genotypes A and D, with a lower prevalence of types F and G, are the most frequent in Europe[51]. Migration is changing this distribution[52,53]. In Europe, different genotypes may play a role in establishing the response to treatment, the manner and time of transmission, and the rate of HBeAg seroconversion with or without therapy[54]. HBV genotype may be associated with disease progression[55].

PROBBABILITY OF RESPONSE TO TREATMENT AND APPEARANCE OF RESISTANCE

Treatment is indicated if there is a high risk of liver-related mortality and morbility and a high likehood of manteined viral supression after a defined course of therapy[24]. This risk is variable during the course of HBV infection.

There are several treatment strategies for CHB: IFN and PEG-IFN, lamivudine, adefovir dipivoxil,

telbivudine and entecavir. None of theses achieves complete HBV eradication and they have limited long-term efficacy. In the majority of patients, particularly those with HBeAg-negative disease, HBV is suppressed but not eradicated by treatment, and relapses occur when drug treatment is interrupted.

For HBeAg-positive patients, the likelihood of a sustained virological response to antiviral therapy is < 20%-30% of treated patients, but long-term response depends upon HBeAg seroconversion. IFN has proven to be efficacious for ALT and DNA normalization, and for HBeAg seroconversion in 25%-40% of HBeAg-positive patients. HBeAg seroconversion is sustained in < 20% at 12 mo. After HBeAg seroconversion is achieved, viral suppression is sustained in 50%-90% of patients[56,57]. PEG-IFNα2A combined response (HBeAg and DNA suppression, and ALT normalization) is better than IFN response at 6 mo (24% *vs* 12%)[58,59]. For NAs, HBeAg/anti-HBeAg seroconversion rate is 16%-24%, and is similar among different drugs[60-63]. These patients have a sustained remission even after therapy is stopped. Entecavir seroconversion rates increase in time but there are no studies after 3 years of treatment, therefore, we do not know if these responses will be maintained[64,65].

For HBeAg-negative patients, the sustained response is low: 15% show normalization of serum ALT and suppression of serum HBV DNA[66-69]. For HBeAg-positive patients, the likelihood of response to nucleoside64/nucleotide analogs[70-74] and IFN[75,76]/PEG-IFN[77] depends greatly upon degree of serum aminotransferase elevation. In general, treatment with any of these drugs does not result in higher rates of HBeAg seroconversion compared to non- treatment in those who have a serum ALT < 2 × ULN. After a year of treatment, HBeAg seroconversion occurs in < 10% of these patients treated with IFN, lamivudine or adefovir. ASSLD guidelines do not recommend treatment in normal ALT patients, because it is unlikely to achieve HbeAg seroconversion.

IFNs are administered for predefined durations, do not select antiviral-resistant mutants, but produce more secondary effects (5%-8% treatment withdrawal). NA oral agents are administrated until specific endpoints are achieved. NA treatment is costly and their long-term safety and efficacy have yet to be proven. The HBeAg-positive patients with normal/low pre-treatment ALT level must not be treated with IFNs because the sustained response is very low. This subgroup and HBeAg-negative patients need long-term or continuous treatment. The hardest challenge for NAs is the selection of antiviral-resistant mutants, with long-term treatment. The rate at which resistant mutants are selected is related to pre-treatment serum HBV-DNA level, speed of viral suppression, duration of treatment, and prior response to NA therapy. Therefore, resistance is greater in normal-level ALT, HBeAg-positive patients, because slower DNA suppression occurs in the first 6 mo of treatment (12 mo for adefovir)[78]. In patients without an early virological decrease (3 and 6 mo), treatment should be maintained for the long term, which does increase the risk of resistance developing.

Lamivudine has the higher rate of resistance, 70% after 4 years of treatment in both HBeAg-positive/negative patients. Adefovir and telbivudine have the same problem. Adefovir resistance in the first year is lower than lamivudine resistance, but later, the accumulated resistance is of the order of 2.5% per year. Telbivudine selects the same resistant mutants as lamivudine. Entecavir-resistant mutation is observed in < 1% of nucleoside-naive patients after 2 and 3 years of treatment; data are lacking after 3 years, although resistance is higher in lamivudine-refractory patients[79,80]. The most effective method against the development of antiviral-resistant HBV is not to treat if therapy is not indicated.

Many new treatments are undergoing testing. None of the combination therapies has been proven to be superior to monotherapy, but with some disadvantages, such as greater cost, toxicity and drug interactions. Finally, current treatments do not eradicate HBV and have limited long-term efficacy, especially if ALT is below two times the normal upper average limit. For HBeAg-negative patients, prediction of response is unclear. While IFNs are administered for predefined durations, NAs are usually administrated until specific endpoints. Treatment is long term in normal-level ALT, HBeAg-positive and -negative patients, and is associated with adverse events and drug resistance.

CONCLUSION

DNA viral load is associated with disease progression, cirrhosis and HCC. In the subgroup of HBeAg-positive patients with DNA levels > 20 000 IU/mL and normal (or minimally elevated) ALT, current therapies are of limited efficacy and treatment should be considered long-term. The risk of this attitude is the appearance of secondary effects and drug resistance. Therefore, viral load is not sufficient for treatment, and we must give some thought to other factors, such as histological factors (fibrosis/cirrhosis and liver inflammation), patient age, disease evolution time, family history of HCC, adverse drug events and development of resistance. If patients have high DNA levels and normal ALT, without other unfavorable prognostic factors, it is advisable to follow the patients and not to treat them. The fluctuations in viral DNA, ALT and liver histology in patients with CHB allow us to choose the best time to begin treatment. The time required for the best response following clinical guidelines may be with the same as that required for the best sustained response. In HBeAg-negative patients, the cut-off value of viral load needed to distinguish asymptomatic HBeAg-negative CHB patients from those with normal ALT and inactive carrier state is not known.

Therefore, it is not the time to treat CHB patients, guided only by viral load. We agree with the clinical practice guidelines of the European Association for the Study of the Liver)[81], in which there are three criteria

for beginning HBV therapy: serum HBV-DNA levels, serum aminotransferase levels, and histological grade and stage. Patients should be considered for treatment when HBV-DNA levels are > 2000 IU/mL (10 000 copies/mL) and/or serum ALT is above ULN, and liver biopsy (or non-invasive markers when validated) shows moderate to severe active necro-inflammation and/or fibrosis (greater than A2 or F2 by METAVIR histological scoring). This may change if new, short-term therapies appear, and HBV eradication should be possible during long-term treatment, without development of resistance.

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Reversal of multi-drug resistance by pSUPER-shRNA-mdr1 *in vivo* and *in vitro*

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Abstract

AIM: To explore the possibility of reversing multi-drug resistance (MDR) to HepG2/mdr1 *in vitro* and *in vivo* with RNA interference (RNAi).

METHODS: HepG2/mdr1 was obtained by cloning the whole gene mdr1 into HepG2 cells. shRNA targeting sequence was designed to be homologous to the P-gp encoding MDR1 mRNA consensus sequence. pSUPER-shRNA/mdr1 was constructed using the enzyme-digested technique. HepG2/mdr1 cells were transfected with vectors of pSUPER-shRNA/mdr1 to measure their efficacy by real-time PCR for mdr1 mRNA, flow cytometry (FCM) for P-gp expression, and Rhodamine efflux, MTT method for HepG2/mdr1 function, respectively. *In vivo*, mice tumors were treated by injecting pSUPER-shRNA/mdr1 *in situ* and into intra-abdominal cavity. Tumors were collected to create cell suspension and cryosections after chemothearpy with adiramycin and mytomycin. The cell suspension was incubated in RPMI-1640 supplemented with G418 to screen stable cells for appreciating the reversal of MDR. Cryosections were treated with immunohistochemistry technique to show the effectiveness of transfection and the expression of P-gp.

RESULTS: pSUPER-shRNA/mdr1 was successfully constructed, which was confirmed by sequencing. The MDR phenotype of HepG2/mdr1 was decreased significantly *in vitro* transfection. HepG2/mdr1 showing its MDR was reversed notably in P-gp expression (11.0% *vs* 98.2%, $P < 0.01$). Real-time PCR showed that mRNA/mdr1 was lower in test groups than in control groups (18.73 ± 1.33 *vs* 68.03 ± 2.21, $P < 0.001$). Compared with HepG2, the sensitivity of HepG2/mdr1 and HepG2/mdr1-dsRNA cells to ADM was decreased by 1.64 times and 15.6 times, respectively. The accumulation of DNR in positive groups was decreased evidently. *In vivo*, the p-gp expression in positive groups was significantly lower than that in control groups (65.1% *vs* 94.1%, $P < 0.05$). The tumor suppressing rate in test groups was 57.8%. After chemotherapy, the growth rate in test groups was lower than that in control groups (700.14 ± 35.61 *vs* 1659.70 ± 152.54, $P < 0.05$). Similar results were also observed under fluorescence microscope, and confirmed by Image-Pro Plus 4.5 analysis.

CONCLUSION: pSUPER-shRNA/mdr1 vector system allows simple, stable and durable nonviral knockdown of P-gp by RNAi in malignant cells and animals to restore their sensitivity to adriamycin.

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Key words: Hepatocellular carcinoma; Multi-drug resistance; RNA interference

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INTRODUCTION

It has been reported that a relatively new technique using RNA interference (RNAi) is able to inhibit gene expression[1]. RNAi is triggered by the presence of

double-stranded RNA (dsRNA) in cells and results in a rapid destruction of mRNA containing an identical or nearly identical sequence[2]. Studies on the RNAi pathway in invertebrates, however, have demonstrated that cleavage of long dsRNAs to a length of 21-25 bp results in dsRNAs that are active in eliciting RNAi[3,4]. It has recently been shown that RNAi can be achieved in cultured mammalian cells using small interfering RNA (siRNAs) with a length of 21-23 bp[5].

Liu *et al*[6] have developed a technique to efficiently deliver plasmid DNA to organs of postnatal mice by rapidly injecting a large volume of physiological solution into their tail vein. A further research showed that high-pressure delivery technique can deliver siRNAs for RNAi induction used as a reporter gene, the modified luciferase gene, luc+[7]. In liver, injection of 0.5 μg siRNA-luc+ can increasingly inhibit luc+. These results indicate that inhibition of target gene expression by siRNA in organs of postnatal mice can efficiently deliver siRNA.

Resistance to cytotoxic drugs represents a serious hindrance in cancer chemotherapy. One form of multi-drug resistance (MDR) is caused by over-expression of P-gp, a mdr1 gene product[8]. P-gp is a transmembrane phosphoglycoprotein capable of transporting a variety of structurally and functionally diverse chemotherapeutic drugs, such as vinblastine, doxorubicin and paclitaxel, leading to reduced intracellular drug concentration and cytotoxicity[9]. In human hepatocellular carcinoma (HCC), the best treatment is radical operation of the tumor. However, only 30%-40% of HCC can undergo a radical operation, while others must receive chemotherapy[10]. To circumvent the MDR of HCC resulting from chemotherapeutic drugs, several ways including inhibitors of P-gp, oligonucleotide, anti-sense RNA, transient siRNA, hammerhead ribozyme techniques and others regulating MDR-related gene measurements have been developed[11-16]. However, the half-life of P-gp (at least 16 h) makes it difficult to achieve a complete knockdown of P-gp[17]. To solve this problem, siRNA vectors have been used in retro-, adeno-, and lentiviral systems. Virus-associated difficulties, such as insertional mutagenesis and cis-activation of silent genes by their strong promoters, and difficult generation of recombinant viruses, have limited the use of such systems[18,19].

Reports showed that RNAi technology is currently used not only as a powerful tool for analyzing gene function, but also for developing highly specific therapeutics[20,21]. In the RNAi approach, sequence-specific post-transcriptional gene silencing is achieved by siRNA, short double-stranded RNA molecules in which the anti-sense strand is complementary to the target mRNA of a given gene[22,23]. It has not been determined whether suppressing mdr1 expression with siRNA is sufficient to reverse the MDR of HepG2/mdr1 *in vitro* and *in vivo*.

An alternative new strategy is the plasmid-based expression system. We used the plasmid-based RNAi system for the knockdown of MDR-1, restoring its sensitivity to adriamycin and doxorubicin in P-gp over expression. This vector system-pSUPER is a powerful tool for the attenuation of P-gp-mediated drug resistance in malignant cells.

The effective dsRNA sequence has been screened from 4 siRNA transfecting HepG2/mdr1 cells with an oligofectamine kit (result not shown). In the present study, we used this sequence in design of shRNA template oligonucleotide.

MATERIALS AND METHODS

Cell culture

HepG2/mdr1 was obtained by cloning the whole gene mdr1 into HepG2 cells to construct its MDR[24]. HepG2 and HepG2/mdr1 cells were cultured in RPMI-1640 containing 2% glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal calf serum, and 200 μg/mL G418 at a density of 2×10^5 and sub-cultured upon reaching a density of 1×10^6 cells per Ml. Three days before transfection, G418 was omitted from the medium.

Design of shRNA template oligonucleotides and construction of plasmid

ShRNA target sequence was designed to be homologous to the P-gp encoding MDR1 mRNA consensus sequence (GeneBank accession number AF 16535) tested by transfection assay[24]. Complementary oligonucleotides encoded a hairpin structure with a 19-mer stem derived from the mRNA target site. A 19-bp loop sequence separated the two complementary domains. Near the 3' end of shRNA template is a 5 nucleotide poly (T) tract recognized as a RNA pol Ⅲ termination signal. The 5' end of two oligonucleotides was Bal Ⅱ and Hind Ⅲ restriction site overhangs. shRNA insert template oligonucleotides, MDR1 DNA oligos, control insert sequence DNA oligos encoding the GFP shRNA (sense: 5'-GATCCGGTTATGTACAGGAACGCATTCAAGAGATGCGTTCCTGTACATAACCT-TTTTGGGAAA-3' and anti-sense: 5'-AGCTTTTCCAAAAAGGTTATGTAC-AGGAACGCATCTCTTGAATGCGTTCCTGTACATA-ACCG-3') were synthesized, annealed and ligated into the Bal Ⅱ and Hind Ⅲ sites of linearized pSuper RNA system shRNA expression vector for each target site.

Cloning shRNA insert into pSUPER neo vector

Ligation products were transformed into *E. coli*, clones were selected, and plasmid DNA was isolated. Plasmid was digested, clones with the shRNA insert were selected, and purification of pSUPER-neo shRNA plasmid for transfection was performed using the Wizard plus SV Minipreps DNA purification system (Promega, USA).

Cell lines and culture

HepG2/mdr1 was kindly provided by Dr. Yong-Bing Chen (Department of Biliary-hepatology Surgery, Beijing Youan Hospital, Beijing 100000, China). All

cells were cultured in RPMI-1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were plated in a fully humidified atmosphere containing 5% CO_2, 95% air at 37℃. HepG2/mdr1 cells were established by gene clone technique. In brief, the mdr1 gene was cloned into the vector and then transfected into HepG2 cells to construct its MDR[20]. In order to maintain the MDR phenotype, cell culture medium for P-gp-expressing HepG2/mdr1 cells was supplemented with 0.05 μg/mL of doxorubicin, 400 μg/mL of G418. HepG2/mdr1 cells were incubated in a doxorubicin-free medium with 400 μg/mL of G418 for over 2 wk and then used in study. All the cells were discarded after 3 mo and new cells were obtained from frozen stocks.

Transfection with siRNA expression vector

HepG2/mdr1 cells in the exponential phase of growth were plated in 6-well plates at 2×10^5 cells per well, grown for 24 h, then transfected with siRNA expression plasmid pSUPER-shRNA (OligoEngine Co, USA) according to the manufacturer's protocol. Negative control cells were treated with pSUPER negative control plasmid consisting of a circle plasmid encoding a siRNA whose sequence was not found in mouse, human or rat genome databases. Seventy-two hours after transient transfection, silencing was examined, then the cells were trypsinized for 72 h and seeded onto selective media. HepG2/mdr1 cells stably expressing siRNA were established by selection with a medium containing 400 μg/mL of G418 (Sigma, USA). The medium was renewed every 3 d. After 2 wk, resistant colonies were trypsinized, combined in pools and cultured in a selective medium. Stable expression was examined 8 wk after transfection.

Real-time fluorescence quantitative PCR

Sequences of the primers and probes were selected from the mdr1 gene for MDR and P-glycoprotein. The primers and TaqMan probes were designed using the Primer Express Software (Applied Biosystems, Foster City, CA). Molecular beacons were designed according to the guidelines available at internet. A DNA folding program was used to estimate the stability of stem and loop structures of the molecular beacons. The forward and reversed primer sequences for mdr1 gene and GAPDH are 5'-GACATGACCAGGTATGCCTA-3' and 5'-CTTGGAGACATCATCTGTAAGTC-3', and 5'-TGGGTGTGAACCACGAGAA-3' and 5'-GGCAT GGACTGTGGTCATGA-3', respectively. The primer sequences of TaqMan probes for mdr1 and GAPDH are 5'-CTGCACCACCAACTGCTTAGC-3' and 5'-CT GACTCACCACACCAATGAC-3', respectively. First, total RNA was extracted from all groups with a RNA extraction kit (Omega, USA) and verified by 1% gel electropherogram and A260/A280. Reverse transcription PCR was carried out to produce cDNA. PCR was performed in a 50 μL volume containing 10 × buffer, 25 mmol/L $MgCl_2$, 25 mmol/L dNTP mix, 10 μmol/L primer F, 10 μmol/L primer R, Taq DNA polymerase and DEPC-H_2O. The reactions were initially heated to 94℃ for 4 min, and then subjected to 35 cycles at 94℃ for 30 s, at 55℃ for 30 s, at 72℃ for 1 min, at 72℃ for 1 min in an iCycle iQ PCR detection system (Bio-Rad, CA). Finally, real-time PCR was performed in a 30 μL volume containing 10 × buffer, 10 mmol/L dNTPs, 250 mmol/L $MgCl_2$, 10 Pmol/μL primer F, 10 Pmol/μL primer R, 10 Pmol/μL Taq Man probe, Taq enzyme, cDNA template and ddH_2O, for 35 cycles at 94℃ for 2 min, at 94℃ for 20 s, at 50℃ for 30 s, and at 60℃ for 40 s for the measurement of mdr1 mRNA level. Data were collected during reactions. PCR threshold cycle (Ct) defined as the fractional cycle number at which the fluorescence reaches 10 times the standard deviation (SD) of the baseline, was determined. Average Ct for duplicate standards and test samples was calculated. Standard curve equations were calculated by regression analysis of average Ct *versus* log10 of the standard copy number. ΔCt and ΔΔCt of all samples were calculated to produce $2^{-\Delta\Delta Ct}$. The results were linearized and analyzed by ANVON.

Flow cytometry (FCM) for P-gp expression

Nucleofected cells of HepG2/mdr1, including test groups-HepG2/mdr1-si and control groups-HepG2/mdr1 were collected, analyzed for Pgp expression with staining 50 μL of Pgp monoclonal antibody for 60 min at 37℃, and washed two times in phosphate-buffered saline (PBS). The cells were incubated fro another 60 min with 50 μL of PE-labled antibody against Pgp antibody, washed two times with PBS, and finally, analyzed with a flow cytometer (FACSCalibur, BD, Vienna, Austria). The data were then subjected to CellQuest software (BD).

Cytotoxicity assay for cell survival

All cells in the exponential growth phase were plated in a 96-well plate at 2×10^4 cells/well, and grown for 24 h. The cells were incubated for 48 h after different concentrations of doxorubicin were added. The cells were incubated for 4 h after 20 μL of 5 mg/mL MTT (Sigma, USA) was added. The absorbance at 490 nm was read and the IC50 values were determined (at least three times) in multiple independent experiments. Relative reversal rate = $(IC_{50}A-IC_{50}B)/(IC_{50}A-IC_{50}C)$, where $IC_{50}A$ is IC_{50} values for non-transfected multi-drug resistant cells, $IC_{50}B$ is IC_{50} values for sensitive cells. Survival data of cells transfected with shRNA vectors were evaluated by ANOVA for statistical significance.

FCM for accumulation of daunorubicin

To assess the steady accumulation of daunorubicin, all cells transfected with shRNA expression plasmids or control plasmids and transfected reagents were incubated with 1.0 μg/mL daunorubicin for 1 h at 37℃, washed three times with ice-cold PBS. The fluorescence intensity of intracellular daunorubicin was determined by FCM.

Subcutaneous tumorigenesis in nude mice

Animal care and sacrifice were approved by Sichuan University Medical School Animal Studies Committee. Prior to injection, HepG2/mdr1 and HepG2 cells were resuspended in a serum-free medium at 2×10^7 cells/mL. Nude mice (BALBC/C-nu/nu, Animal Breeding Center, Sichuan University, Chengdu, China) were divided into experimental group and control group. Mice in each group were subcutaneously injected with 200 μL cell suspension into the right flank. The process of tumorigenesis was monitored and newly formed tumor volume (volume = $m_1^2 \times m_2 \times 0.5236$, m_1 represents the short axis, and the m_2 long axis) was measured every 2-3 d.

In situ and intraperitoneal injection of pSUPER-shRNA-mdr1

When the newly formed subcutaneous tumor reached 0.5-0.6 cm in diameter, mice in the experimental group underwent 2 variant tests (5 mice per test): pSUPER-shRNA-MDR1 at the ratio of 1 mg/kg and 1.5 mg/kg was injected into the tumor *in situ* and peritoneal cavity, respectively. Mice in the negative control group were injected with blank pSUPER vectors and mice in the blank control group of HepG2 were not treated. The vectors were mixed with liposome at the ratio of 1:1 for transfection. Seventy-two hours after injection, mice in all groups underwent chemotherapy with adriamycin (ADM) at a dose of 1.5 mg/kg, twice a week, for two weeks. Then, tumorigenic tissue was carefully separated from its surrounding fibrous capsule for measurement of tumor volume. The relative growth rate of tumorigenic tissue [$\Delta TV = (V_2 - V_1)/V_1$, V_1: tumor volume before chemotherapy; V_2: tumor volume at mouse sacrifice] demonstrated the sensitivity of variant cells to ADM *in vivo*. Tumorigenic tissue was further ground, suspended as single cells, and cultured in RPMI-1640 medium supplemented with 200 μg/mL of G418. Cells with stable expression of shRNA were selected for flow cytometry or incubated with different concentrations of ADM (0.032-5 μg/mL). Their IC_{50} values were determined by MTT assay.

Sensitivity of HepG2/mdr1 to ADM

Seventy-two hours after treatment with pSUPER-shRNA-mdr1 injection, mice in all groups underwent chemotherapy with adiramycin at a dose of 1.5 mg/kg, two times a week, for two weeks, and executed to harvest the tumor for monoplast suspension. The exact volume and relative growth rate of tumor [$\Delta TV = (V_2 - V_1)/V_1$, where V_1 is the tumor volume before chemotherapy and V_2 is the tumor volume after mouse sacrifice] were measured to evaluate drug sensitivity of cell lines to ADM. Cell suspension was cultured in a RPMI-1640 medium supplemented with 200 μg/mL of G418 to screen stably expressing cells. The screened cells were incubated at 1×10^6 cells/mL to study the sensitivity of HepG2/mdr1 to ADM. Cells were treated at different concentrations of ADM (0.032-5 μg/mL) to determine their IC_{50} by MTT assay.

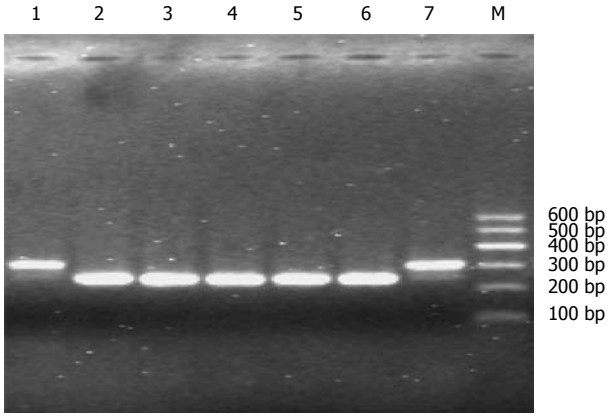

Figure 1 shRNA/mdr1 expression plasmids digested by restriction enzyme Bag Ⅱ and Hind Ⅲ, and a fragment produced in 1.5% agarose gel electrophoresis. Lanes 1 and 7: positive clone size of 310 bp (insert element 64 bp).

Flow cytometry for P-gp expression

Variant cells were incubated with 50 μL of monoclonal antibody to P-gp at 37℃ for 60 min, washed twice in phosphate-buffered saline (PBS), incubated for another 60 min with 50 μL of PE-labeled secondary antibody, sorted with a flow cytometer (FACSCalibur, BD, Vienna, Austria) and analyzed using CellQuest software (BD).

Immunohistochemistry

Sections of tumorigenic tissue were fixed in cold acetone at 4℃ for 10 min. The sections were incubated for 10 min at room temperature with an endogenous peroxidase blocking solution, and then with 1:100 anti-P-gp mouse monoclonal antibodies at 37℃ for 1 h. After washed with PBS, the cells were incubated with 1:150 PE-labeled rabbit anti-mouse IgG at 37℃ for 1 h, stained with glycerine and photographed under inverted light microscope (Olympus, Japan) and confocal fluorescence microscope (Olympus, Japan). The images were analyzed with Image-Pro Plus 4.5 system (Media Cybernetics, Inc. USA). The total OD value and area of intracellular fluorescence for each section were measured.

Statistical analysis

Average values were expressed as mean ± SD. The statistical significance of differences in mean values was assessed by Student's *t* test and ANVON using SPSS 10.0 statistical software. $P < 0.05$ was considered statistically significant.

RESULTS

Identification of shRNA expression plasmids

The shRNA/mdr1 expression plasmids were digested by restriction enzyme Bag Ⅱ and Hind Ⅲ, and 1.5% agarose gel electrophoresis produced a fragment of 64 bp (Figure 1). DNA sequencing confirmed that ligation reaction between the shRNA insert and pSUPER-shRNA/mdr1 expression vector was correct. No.1 and No.7 indicated the positive clone size of 310 bp (insert element 64 bp) (Figure 2).

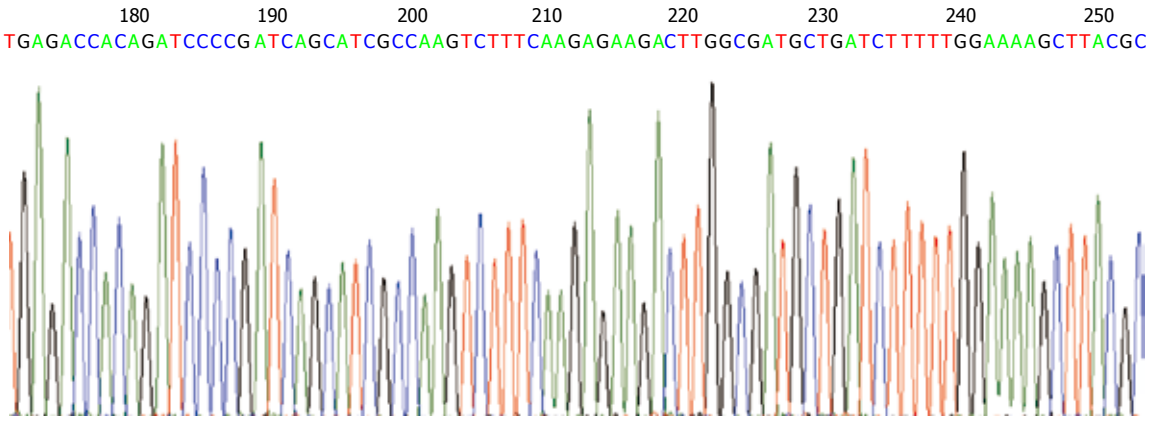

Figure 2 Sequence of pSUPER-shRNA/mdr1 (inserted element 64 bp).

Table 1 Effect of stable expression of MDR1 targeting shRNA on MDR1 mRNA, P-gp and resistance to cytotoxic drugs in HepG2/mdr1 cells

	Real-time PCR mdr1 mRNA	P-gp	DNR accumulation	Drug resistance			
				Adiramycin		Mytomycin	
Cell line	$2^{-\Delta\Delta Ct}$	Mean fluorescence	Mean fluorescence	IC_{50}	Fold-resistance	IC_{50}	Fold-resistance
HepG2	75.58	1.0	87.56	0.40	1.0	0.07	1.0
HepG2/m	1.0	98.6	39.76	25.0	62.5	9.67	138.1
HepG2/m-sh	56.30[b]	1.07[b]	79.32[a]	0.56[b]	1.4[b]	0.08[b]	1.14[b]

The unit of IC_{50} was μg/mL. [a]$P < 0.05$; [b]$P < 0.01$ *vs* HepG2/m.

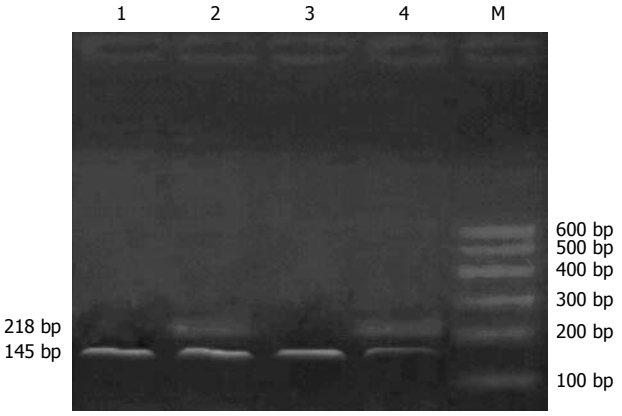

Figure 3 Real-time PCR showing significantly reduced mdr1 mRNA expression in cells treated with pSUPER-shRNA/mdr1 vector. 1: HepG2; 2: HepG2/mdr1; 3: HepG2/m-sh; 4: HepG2/mdr1 empty vector; M: Marker; Mdr1: 218 bp; GAPDH: 145 bp.

Inhibition of mdr1 gene mRNA expression using shRNA expression vectors

Seventy-two hours after transient transfection, mdr1 mRNA expression was significantly reduced in cells treated with pSUPER-shRNA/mdr1 vector, compared with the control (56.3-fold *vs* 1.0-fold, $P < 0.01$; Figure 3 and Table 1).

Knockdown of P-gp protein expression after transfection with pSUPER-shRNA/mdr1 vectors

The effect of transfection with pSUPER-shRNA/mdr1 vectors on cellular P-gp expression in HepG2/mdr1 cells was assessed by FCM. Seventy-two hours after transient transfection with pSUPER-shRNA/mdr1 vectors, the protein expression of P-gp was decreased from 98.6% to 11.0% ($P < 0.01$, Table 1 and Figure 4), indicating that transfection with pSUPER-shRNA/mdr1 vectors could significantly decrease the cellular P-gp expression levels in HepG2/mdr1 cells after transient and stable transfection.

Cell survival

MTT assay was performed to examine the effects of doxorubicin after transient and stable transfection. Seventy-two hours after transient transfection, pSUPER-shRNA/mdr1 vectors decreased the resistance to adiramycin and mytomycin from 44.6-fold to 1.4-fold and from 138.1-fold to 1.14-fold compared to the control groups (Table 1 and Figure 5). The IC50 was lower than that in control groups (0.56 μg/mL *vs* 25.0 μg/mL, 0.08 μg/mL *vs* 9.67 μg/mL, $P < 0.01$), suggesting that transfection of pSUPER-shRNA/mdr1 vectors could reverse the resistance to adiramycin and mytomycin.

Steady accumulation of daunorubicin

Transfection with hairpin shRNA vectors improved intracellular drug accumulation. Compared with control groups, accumulation of daunorubicin in cells treated with shRNA vectors was significantly increased (79.32% *vs* 37.96%, $P < 0.05$). There was no difference in sensitive cells of HepG2 (79.32% *vs* 87.56%) between test groups (Table 1 and Figure 6).

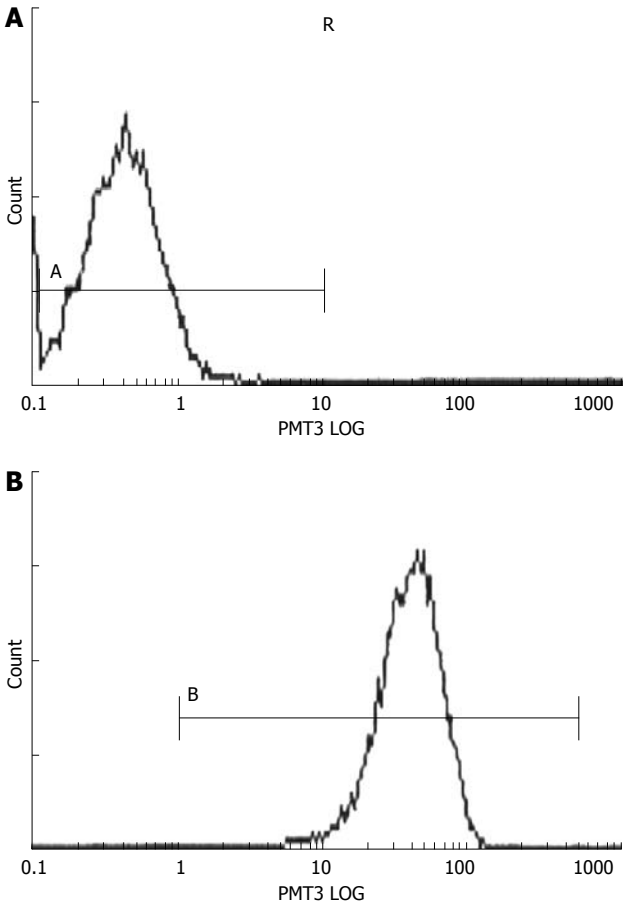

Figure 4 FCM showing P-gp expression of HepG2/mdr1 in test groups (A) and control groups (B).

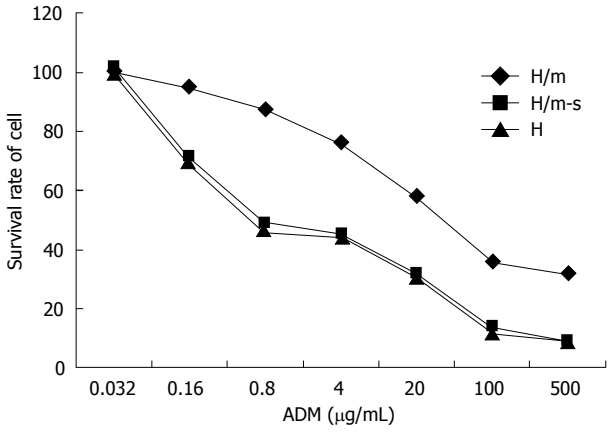

Figure 5 Sensitivity of HepG2/mdr1 and HepG2/mdr1-siRNA to ADM. After transfected with pSUPER-shRNA/mdr1 vectors, the resistance of HepG2 cells to adiramycin and mytomycin decreased from 44.6-fold to 1.4-fold and from 138.1-fold to 1.14-fold.

Establishment of tumor implants

Two weeks after HepG2/mdr1 and HepG2 cells were injected into the right flank of 4-wk-old nude mice, all mice suffered from tumors measuring 0.5 cm × 0.6 cm × 0.6 cm to 0.6 cm × 0.6 cm × 0.65 cm (Table 1). There was no difference in the mean tumor volume between HepG2/mdr1 and HepG2 groups (64.23 ± 2.15 mm^3 *vs* 66.41 ± 1.58 mm^3).

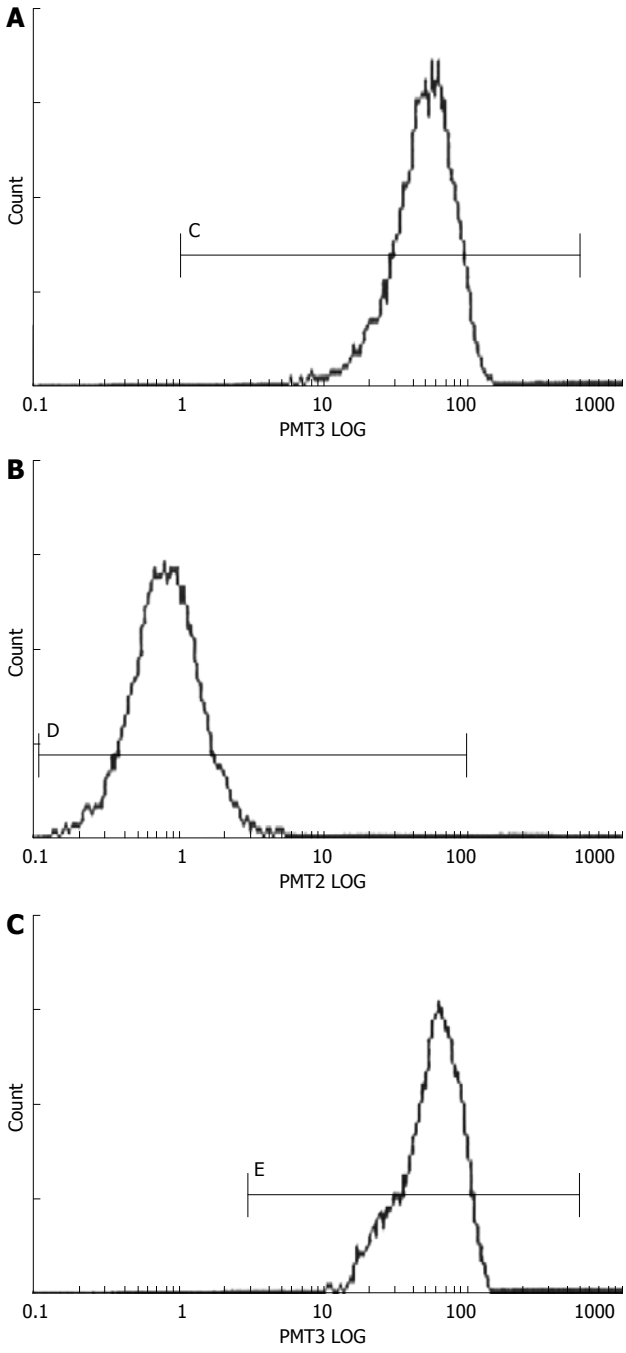

Figure 6 DNR accumulation of HepG2/mdr1 (A, B) and HepG2 cells (C) pre- and post-transfection. Compared with control groups, accumulation of daunorubicin in cells treated with shRNA vectors increased significantly (79.32% *vs* 37.96%, $P < 0.05$). There was no difference in sensitive cells of HepG2 between test groups (79.32% *vs* 87.56%).

Changes in tumor volume after treatment with ADM

After treatment with ADM, changes of tumor volume were more significant in the transfected groups than in the control groups (1695.70 ± 152.54 mm^3 *vs* 700.14 ± 25.61 mm^3, $P < 0.01$; Figure 7, Table 2). *In vivo* implant of HepG2/m-sh was more sensitive to ADM than that of HepG2/m. However, the difference in ADM sensitivity between *in vivo* implant of HepG2 and HepG2/m-sh was not significant. The results demonstrate that tumor cells in transfected groups could reverse their sensitivity to ADM.

Table 2 Effect of expression of MDR1 targeting shRNA on P-gp expression, tumor growth and adiramycin sensitivity *in vivo*

Group	Mean growth time (d)	Mean volume (mm^3) pretreatment	P-gp expression	ΔVolume (mm^3) pre- & post-chemotherapy	Growth rate of tumor
HepG2	13.12 ± 1.35	66.41 ± 1.58	-	530.58 ± 23.12	8.46 ± 0.63
HepG2/m	12.65 ± 2.31	64.23 ± 2.15	97.90[b]	1659.70 ± 152.54[b]	23.12 ± 5.32[b]
HepG2/m-sh	-	-	15.75	700.14 ± 25.61	10.39 ± 1.54

[b]$P < 0.01$ *vs* HepG2/m-sh.

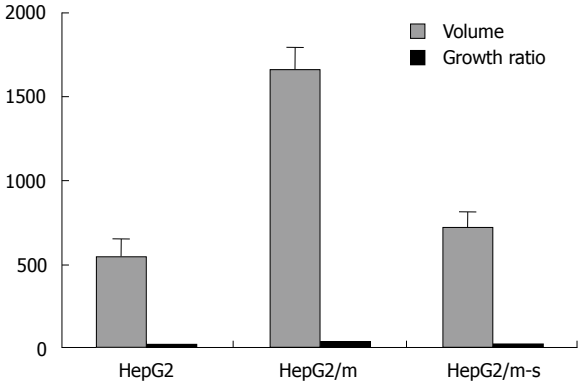

Figure 7 Changes in volume and growth ratio of tumor after treatment with ADM. More changes in tumor volume occurred in transfected groups than in control groups (1695.70 ± 152.54 mm^3 *vs* 700.14 ± 25.61 mm^3, $P < 0.01$).

FCM for P-gp expression in mice

P-gp expression was detected by FCM after the mice were sacrificed. P-gp expression in test groups was lower than that in control groups (65% *vs* 94.1%, $P < 0.05$), but there was no significant difference between groups treated with *in situ* and intra-abdominal injection of pSUPER-mdr1 (65.1% *vs* 58.7%), showing that effect of pSUPER-shRNA-mdr1 could be achieved both by *in situ* injection and by intra-abdominal injection of pSUPER-mdr1 (Figure 8).

Immunohistochemistry

After chemotherapy, nude mice were sacrificed the next day. Tumors were collected by cervical dislocation or by drawing blood from heart, and cryo-sectioned (8 μm). The sections were treated with immnohistochemistry and analyzed by Image-Pro Plus 4.5 software (Media Cybernetics, Inc USA). The density of green fluorescence represented the tranfected vector pSUPER-shRNA/mdr1 into tumor. The density of red fluorescence was equal to the amount of P-gp antibodies binding to cellular membrane. The transfection ratio of test groups was greater than that of control groups (86.70% *vs* 35.20%, $P < 0.05$). The expression of P-gp in test groups was lower than that in control groups (15.75% *vs* 97.90%, $P < 0.05$). However, there was no difference in expression of P-gp between *in situ* and intra-abdominal injection of pSUPER-shRUA/mdr1 (97.90% *vs* 92.62%, Figure 9).

DISCUSSION

In cancer, over-expression of mdr1 P-glycoprotein (ABCB1) is a possible cause of chemotherapy-based treatment failure[25]. P-glycoprotein confers cross-resistance to unrelated drugs that differ widely in molecular structure and target specificity, including natural agents and new anticancer agents[26].

Of the novel strategies to circumvent MDR, hammerhead ribozymes against the mdr1 mRNA and anti-sense oligonucleotides have been widely used[12-15]. Both hammerhead ribozymes and anti-sense must be delivered across the plasma membrane, and are susceptible to degradation. Recent studies exploiting the promise of RNAi in mammalian cells have led to the reversal of gene-dependent MDR using short hairpin RNA expression vectors[27,28]. *In vitro* study has not achieved complete reversal of MDR with RNAi[29]. Use of shRNAs, when

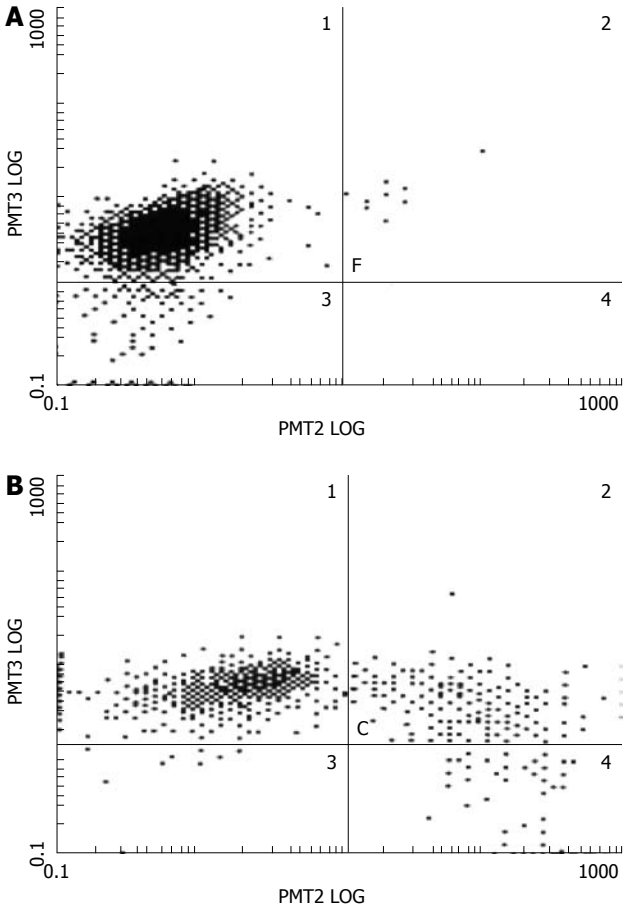

Figure 8 FCM showing lower P-gp expression in HepG2 cells (A) and HepG2/mdr1-shRNA (B) in test groups than in control groups (65% *vs* 94.1%, $P < 0.05$). There was no significant difference between groups treated with *in situ* injection and intra-abdominal injection of pSUPER-mdr1 (65.1% *vs* 58.7%).

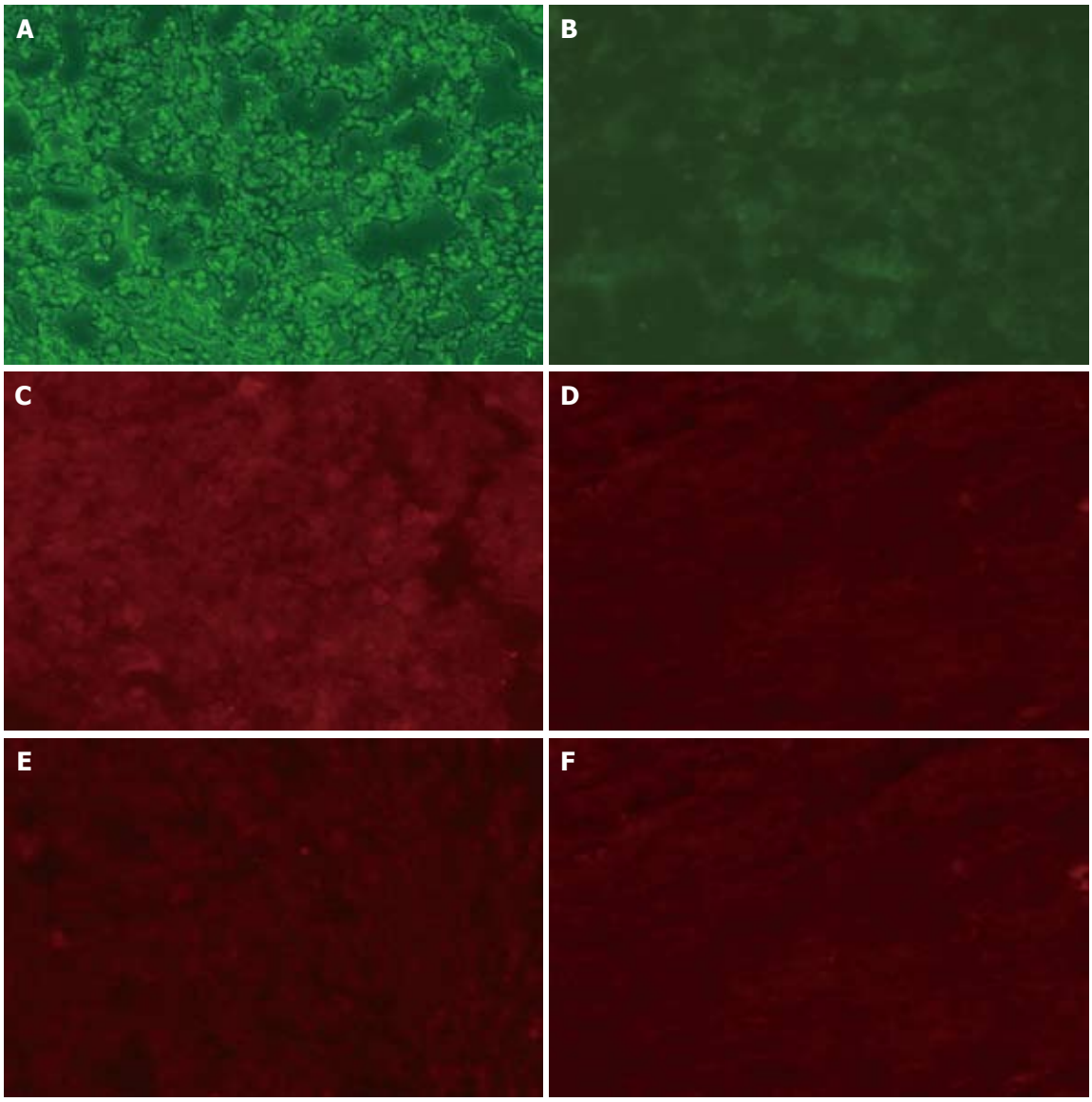

Figure 9 Immunohistochemistry showing green fluorescence in test groups (A) and control groups (B), P-gp expression in test groups (C) and control groups (D), P-gp expression in test groups transfected with *in situs* injection (E) and intra-abdominal injection of pSUPER-mdr1 (F) (× 200). The transfection ratio for test groups was higher than that for control groups (86.70% *vs* 35.20%, $P < 0.05$). The expression of P-gp in test groups was lower than that in control groups (15.75% *vs* 97.90%, $P < 0.05$).

expressed in stably integrated plasmids, can decrease mdr1 mRNA by 95%-97% in intact cells and restore drug sensitivity to that of parent cell lines.

In the present study, shRNA expression cassettes targeting mdr1 were used not only in cells in culture, but also in animals. Pichler *et al*[27] reported that gene silencing induced by pSuper and pRetrosuper constructs is specific and potent in cell lines, and stably express shRNAi for several months. It was reported that high-pressure delivery technique of siRNA through tail vein inhibits expression of genes in a specific manner *in vivo*[7]. However, in this study, siRNA delivered with this method was susceptible to degradation, the effect of RNAi only lasted several days, a time frame insufficient for its application.

The potent and ubiquitous gene-silencing mechanism of RNAi is to generate considerable excitement in the fields of molecular biology and gene therapy[30], but delivery is probably the single biggest obstacle to the development of RNAi-based therapeutic agents[31]. Vector-based delivery of shRNA could improve the efficiency of siRNA delivery. pSUPER RNAi system provides a mammalian expression vector that directs intracellular syntheses of siRNA-like transcripts. The vector uses a polymerase Ⅲ H1-RNA gene promoter, since it produces a small RNA transcript lacking a polyadenosine tail and has a well-defined start of transcription and a termination signal consisting of five thymidines in a row (T5). Cleavage of the transcript at the termination site is after the second uridine, yielding a transcript resembling the ends of synthetic siRNAs, which also contain two 3'overhanging T or U nucleotides (nt)[32,33]. The pSUPER RNAi system has been used to induce efficient and specific down-regulation of gene expression[34,35], resulting in functional inactivation of targeted genes. This vector-induced stable expression

of siRNAs mediates persistent suppression of gene expression, allowing analysis of loss-of-function phenotypes that develop over a longer period of time. We used this vector system to deliver shRNA/mdr1. Combined with bioluminescence imaging, pSUPER was easier to be followed, especially *in vivo*. shRNA-mediated down-regulation of P-glycoprotein transport activity both in cultured cells and in tumor implants in living animals could be followed by direct noninvasive bioluminescence imaging using the *Renilla* luciferase fluorophore. *In vitro*, the vector of pSUPER-shRNA/mdr1 was constructed using enzyme-digested technique according to the pSUPER protocol. Transfected with the recombinant plasmid, HepG2/mdr1 showed that its MDR was reversed notably in P-gp expression between positive and control groups (11.0% *vs* 98.2%, $P < 0.01$). Real-time PCR showed that mRNA/mdr1 in test groups was lower than that in control groups (56.30-fold *vs* 1.0-fold, $P < 0.001$). Compared with HepG2/mdr1 cells, the sensitivity of HepG2/mdr1-dsRNA cells to adiramycin and doxorubicin was decreased from 15.6-fold to 1.64-fold and from 138.1-fold to 1.14-fold, respectively. The accumulation of DNR in positive groups was decreased evidently. These results indicate that plasmid vector of pSUPER-shRNA/mdr1 can effectively transfect target cells and suppress gene function. *In vivo*, after transfected with pSUPER-shRNA/mdr1 through the peritoneal cavity and *in situ*, carcinoma cells(HepG2 and HepG2/mdr1) measured by flow cytometry and immunohistochemistry analysis showed that dsRNA/mdr1 was successfully and effectively brought into cells, and that p-gp expression in positive groups was significantly down regulated compared to control groups(65.1% *vs* 94.1%, $P < 0.05$). The tumor suppressing rate for test groups was 57.8%. After chemotherapy, the growth rate of tumor for test groups was slower than that for control groups (700.14 ± 35.61 *vs* 1659.70 ± 152.54, $P < 0.05$). Similar results were also observed under fluorescence microscope, and confirmed by Image-ProPlus 4.5 software analysis.

The results indicate that vector-based delivered RNAi can improve its efficiency *in vitro* and *in vivo*. There was no difference in the efficacy of vector pSUPER-shRNA/mdr1 transfected with *in situ* injection and that intra-abdominal injection (data not shown). Our study showed that transfection of vector pSUPER- shRNA/mdr1 with intra-abdominal injection was effective due to its diffusivity, which can extend from location to system. It was recently reported that administration of RNAi targeted mdr1 gene can effectively reverse MDR both *in vitro* and *in vivo* human epidermoid carcinoma KB(v200) cells[21]. This is the first time to investigate the possibility of reversing MDR in HepG2/mdr1 cells and tumor implants with RNAi.

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COMMENTS

Background

Hepatocellular-carcinoma is very resistant to chemotherapy.There is a growing interest in the reversal of multi-drug resistance to tumors with RNA interfer-ence (RNAi). In hepatocellular carcinoma, such research is almost restricted *in vitro*. In the present study, the authors investigated the effect of RNAi on the reversal of multi-drug resistance to hepatocellular carcinoma *in vitro* and *in vivo*.

Research frontiers

This study focused on the reversal of multi-drug resistance to hepatocellular carcinoma with RNAi *in vivo*.

Innovations and breakthroughs

The present study showed that RNAi targeting mdr1 gene could reverse the resistance of hepatocellular carcinoma to chemotherapy. Transfection with *in situ* injection and intra-abdominal injection showed that RNAi could reverse multi-drug resistance of hepatocellular carcinoma.

Applications

These data demonstrate that RNAi targeting mdr1 gene could reverse drug resistance of hepatocellular carcinoma *in vivo*, thus contributing to the development of drugs used in gene therapy for hepatocellular carcinoma.

Peer review

This is a carefully performed study with novel findings, which can be used in the diagnosis and treatment of hepatocellular carcinoma. The study showed that RNAi targeting mdr1 gene can reverse drug resistance of hepatocellular carcinoma *in vivo*, and therefore improves its sensitivity to chemotherapy for HepG2/mdr1 cells and transplant tumors of HepG2/mdr1.

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ORIGINAL ARTICLES

Suppression of pancreatic carcinoma growth by activating peroxisome proliferator-activated receptor γ involves angiogenesis inhibition

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Abstract

AIM: To study the possible actions and mechanisms of peroxisome proliferator-activated receptor γ (PPARγ), a ligand-activated transcription factor, in pancreatic carcinogenesis, especially in angiogenesis.

METHODS: Expressions of PPARγ and retinoid acid receptor (RXRα) were examined by reverse-transcription polymerase chain reaction (RT-PCR) with immunocytochemical staining. Pancreatic carcinoma cells, PANC-1, were treated either with 9-cis-RA, a ligand of RXRα, or with 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2), a ligand of PPARγ, or both. Antiproliferative effect was evaluated by cell viability using methyltetrazolium (MTT) assay. A pancreatic carcinoma xenograft tumor model of nude mice was established by inoculating PANC-1 cells subcutaneously. Rosiglitazone, a specific ligand of PPARγ, was administered *via* water drinking in experimental group of nude mice. After 75 d, all mice were sacrificed. Expression of proliferating cell nuclear antigen (PCNA) in tumor tissue was examined with immunohistochemical staining. Expression of vascular endothelial growth factor (VEGF) mRNA in PANC-1 cells, which were treated with 15d-PGJ_2 or 9-cis-RA at various concentrations or different duration, was detected by semi-quantitative RT-PCR. Effects of Rosiglitazone on changes of microvascular density (MVD) and VEGF expression were investigated in xenograft tumor tissue. Neovasculature was detected with immunohistochemistry staining labeled with anti-Ⅳ collagen antibody, and indicated by MVD.

RESULTS: RT-PCR and immunocytochemical staining showed that PPARγ and RXRα were expressed in PANC-1 cells at both transcription level and translation level. MTT assay demonstrated that 15d-PGJ_2, 9-cis-RA and their combination inhibited the growth of PANC-1 cells in a dose-dependent manner. 9-cis-RA had a combined inhibiting action with 15d-PGJ_2 on the growth of pancreatic carcinoma. *In vivo* studies revealed that Rosiglitazone significantly suppressed the growth of pancreatic carcinoma as compared to control group (0.48 ± 0.23 cm^3 *vs* 2.488 ± 0.59 cm^3, $P < 0.05$), and the growth inhibition rate was 80.7%. Immunohistochemistry study showed that PCNA was down regulated in Rosiglitazone-treated group compared to the control group. 15d-PGJ_2, 9-cis-RA and their combination inhibited the expression of VEGF mRNA in PANC-1 cells in a dose- and time-dependent manner. MVD was decreased more significantly in Rosiglitazone-treated mice (10.67 ± 3.07) than in the control group (31.44 ± 6.06) ($P < 0.01$). VEGF expression in xenograft tumor tissue was also markedly down-regulated in Rosiglitazone-treated mice.

CONCLUSION: Activation of PPARγ inhibits the growth of pancreatic carcinoma both *in vitro* and *in vivo*. Suppression of tumor angiogenesis by down-regulating the expression of VEGF may be one of the mechanisms by which PPARγ activation inhibits the growth of pancreatic carcinoma.

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Key words: Pancreatic carcinoma; Peroxisome proliferator-activated receptor γ; Angiogenesis; Vascular endothelial growth factor

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INTRODUCTION

Pancreatic carcinoma is currently the fifth most common cause of cancer death in Western Countries[1]. Despite surgical resection, radiotherapy and conventional chemotherapy, the prognosis of advanced pancreatic carcinoma has not been significantly improved over the last 30 years. The inability to detect pancreatic carcinoma at an early stage, its aggressiveness, and lack of effective systemic therapy, are responsible for the rapid death of pancreatic carcinoma patients. Even the recent introduction of deoxycytidine analogue, gemcitabine, does not extend the median survival time of patients with advanced pancreatic carcinoma beyond 6 mo[2]. Clearly, novel approaches to human pancreatic carcinoma therapy are needed.

Pancreatic carcinoma can entail the substantial development of new blood vessels in tumor tissue. It is well established that the growth and progression of solid tumors depend on angiogenesis[3]. Many cytokines or growth factors contribute to angiogenesis. Vascular endothelial growth factor (VEGF) is one of the most important factors.

Peroxisome proliferator-activated receptors (PPARs) are members of the steroid receptor super-family and as such, are ligand-activated nuclear transcription factors[4]. Three subtypes of PPARα, PPARβ (also called δ, NUC-1, or FFAR), and PPARγ have been identified and cloned. Like other members of this super-family, PPARs mediates transcriptional regulation by binding to their central DNA domain that recognizes response elements in the promoters of specific target gene[5]. Activation of PPARγ has been linked to adipocyte differentiation and regulation of glucose homeostasis in humans. Recent tudies also showed that the natural receptor ligand for PPARγ, 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2)[6,7], and synthetic antidiabetic thiazolidinedione drugs, inhibits the activation of macrophages and monocytes[8,9] as well as tumor cell growth[10-12]. PPARγ can heterodimerize with retinoid acid receptor (RXR). For the PPAR: RXR heterodimer, binding of the ligand to either receptor can activate the complex, but binding to both ligands simultaneously is more potent[5]. It has been shown that PPARγ gene expression is observed in a variety of tissues, including adipose tissue and tumor tissue. Some *in vitro* studies have recently reported that PPARγ activation has inhibitory effects on the growth of pancreatic carcinoma cells[13-15], probably due to its up-regulation of cellular apoptosis and its down-regulation of tumor invasion[16-18]. However, little attention has previously been paid to PPARγ action on the growth of pancreatic carcinoma *in vivo*, especially its regulation action on tumor angiogenesis.

In this study, the potent inhibitory effects of PPARγ ligand, 15d-PGJ_2, Rosiglitazone, RXR ligand, 9-cis-retinoid acid (9-cis-RA), on the growth of human pancreatic carcinoma were investigated both *in vivo* and *in vitro*. Expression of proliferating cell nuclear antigen (PCNA) in tumor tissue was also examined. To further clarify the effect of PPARγ on angiogenesis, both *in vivo* and *in* vitro VEGF expression, and neovasculature indicated by microvascular density (MVD) *in vivo* were determined.

MATERIALS AND METHODS

Reagents

15d-PGJ_2 was obtained from Cayman Co (Ann Arbor, MI, USA). 9-cis-RA was from Sigma (St Louis, MO, USA). Rosiglitazone was from SmithKline Beecham Co (Pittsburgh, PA, USA). Anti-PCNA, PPARγ and RXRα polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc (San Diego, CA, USA). Anti-Ⅳ collagen monoclonal antibody was from DAKO Co. (Glostrup, Denmark). Anti-mouse and anti-rabbit detection reagents (HRP) were purchased from Antibody Diagnostica Inc., (Shanghai, China). Oligonucleotides were synthesized by Sangon Co (Shanghai, China). Methyltetrazolium (MTT) and dimethylsulfoxide (DMSO) were purchased from Amresco Inc (Solon, OH, USA).

Cell cultures and treatment

PANC-1 cell line, purchased from American Type Culture Collection (Rockville, MD, USA), was routinely maintained in DMEM containing 10% fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY, USA), 2 mL glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere containing 950 mL/L air and 50 mL/L CO2 at 37℃. PANC-1cells were passaged and expanded by trypsinization of cell monolayers followed by relating every 2 or 3 d.

PANC-1 cells were seeded at a concentration of 5×10^5 cells/well in 6-well plates, and treated with 15d-PGJ_2 and 9-cis-RA and their combination with various concentrations or different duration 24 h later. Cells were then collected for RNA analysis. Control cells were not exposed to the above agents and maintained under the same conditions as the treated cells.

RNA extraction and reverse-transcriptional polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol reagent (Gibco Life Technologies, Inc., Langley, OK, USA) following its manufacturer's instructions. Fist-strand cDNA was synthesized from 3 μg of RNA in 20 μL of reaction solution using a random primer and Superscript Ⅱ reverse transcriptase reagent (Gibco Life Technologies, Inc., Langley, OK, USA). Synthesized cDNA was stored at -80℃ for PCR. The sequences of primers used for amplifying PPARγ, RXRα, and VEGF are 5'-ATGACAGCGACTTGGCAATA-3' and 5'-GCAACTGGAAGAAGGGAAAT-3', 5'-CTCTCAGGTTGAACTCACCT-3' and 5'-ATCTCTGACAGCCTGTCTCG-3', 5'-ATGAACTTTCTGCTGTCTTG-3' and 5'-TGCATGGTGATGTTGGAC-3', respectively. The sequences of GAPDH used as a control are 5'-GTGAAGGTCGGAGTCAACG-3' and 5'-GGTGAAGACGCCAGTGGACTC-3'. After denaturation of the samples at 94℃ for 5 min, 35 cycles were carried out at 94℃ for 45 s, at

55℃ (PPARγ, RXRα) for 45 s, at 54℃ (VEGF) for 45 s, at 60℃ (GAPDH) for 45 s, at 72℃ for 45 s and at 72℃ for 10 min. The PCR products were electrophoresed on 1.5% agarose gels stained with ethidium bromide. The expected molecular size of amplified products was 341 bp of PPARγ, 422 bp of RXRα, 382 bp of VEGF and 223 bp of GAPDH, respectively. Semi-quantitative data about the PCR products were obtained by comparing the intensity of PCR band of VEGF with that of internal control of GAPDH using genetools software.

Immunocytochemistry

Cells were seeded at a concentration of 2×10^5 cells/well in 12-well plates containing slides. Cells were fixed in PBS containin 4% paraformaldehyde (pH 7.4) for 20 min at room temperature, washed 3 times with PBS and incubated with mouse anti-PPARγ (diluted at 1: 150) or rabbit anti-RXRα antibody (diluted at 1: 100) in a humid chamber at 4℃ overnight. Slides were washed with PBS. Secondary antibody (anti-mouse or anti-rabbit antibody) labeled with HRP was applied in humid chambers for 30 min at room temperature. The staining result was detected using a 3, 3'-diaminobenzidine tetrahydrochloride solution (DAB) (DAKO Co., Glostrup, Denmark) for 10 min.

Cell viability assay

PANC-1 cells were seeded at the concentration of 5×10^4 cells/well in 96-well plates and incubated in complete fresh media for 24 h. The cells were subsequently incubated for 48 h and treated either with 0, 2.5, 5, 7.5 and 10 μmol/L of 15d-PGJ_2, or with 0, 5, 10, 15, 20 μmol/L of 9-cis-RA, or with their combination (0, 2.5, 5, 7.5 or 10 μmol/L of 15d-PGJ_2) and (10 μmol/L of 9-cis-RA), respectively. After treatment, MTT solution was added to cells at a final concentration of 500 μg/mL and incubated at 37℃ for an additional 4 h. Then the medium was aspirated, and formazan product was dissplved with DMSO. Cell viability was determined by differences in absorbance at wavelength 490 nm and presented as percentage of control culture conditions.

Animals and tumor cell inoculation

Thirty female BALB/c nu/nu mice (Bikai Experimental Animal Center, Shanghai, China), at the age 6 to 7 wk, were used in the study. The mice were maintained in a laminar airflow cabinet under specific pathogen-free conditions with free access to sterile food and water. For mice inoculation, PANC-1 cells in log-phase growth were harvested by trypsinization, and a medium containing 10% FBS was added. After washed three times with serum-free DMEM, cells were resuspended in phosphate-buffered saline (PBS) at the concentration of 1×10^9/mL, and inoculated subcutaneously into the hindlimb region of each mouse. All experiments were approved by the University Animal Care Committee and carried out according to the National Animal Welfare Law.

Tumor growth in nude mice and Rosiglitazone administration

From the second week of tumor cell inoculation, tumor bearing mice were randomly divided into control groups (n = 15) and Rosiglitazone treatment group (n = 15). Mice in the control group received distilled water and mice in the Rosiglitazone treatment group received Rosiglitazone at the dose of 10 μmol/kg. d. Tumor sizes were measured with a vernier caliper at 25 d intervals and calculated according to the formula: A (length) × B (width) × C (height) × 0.5236. At end of the experiment (75 d), blood was collected for hepatic function analysis. All mice were euthanized and autopsies were performed. Tumors were removed, weighed, and fixed in 10% neural buffered formalin and embedded in paraffin for histological analysis. The growth inhibition rate of Rosiglitazone was calculated.

Immunohistochemistry

Four-μm thick paraffin sections were air-dried, and then placed in a 60℃ oven overnight. The sections were dewaxed in xylene, immersed in a solution of 750 μL 30% hydrogen peroxide and 50 mL methanol for 10 min to block endogenous peroxide, and rehydrated to tap water for antigen retrieval. After boiled in a 10 mmol/L citrate buffer (pH 6.0), slides were incubated overnight at 4℃ with optimum dilutions of primary antibodies, including anti-PCNA mouse polyclonal antibody (× 50 dilution), and anti Ⅳ-collagen mouse monoclonal antibody (× 200 dilution). The sections were washed with PBS and then secondary antibody (anti-mouse antibody) labeled with HRP was applied in humid chambers for 40 min at room temperature. Staining was detected using DAB for 10 min. The sections were counterstained with hematoxylin. Substitution of the primary antibodies with immunoglobin G served as negative controls in all cases.

Measurement of microvessel density (MVD)

Microvessels were shown by staining endothelial cells for Ⅳ collagen using a standard immunoperoxidise technique. MVD was assayed under light microscope. The "*hot spot*" area of neovascularization was identified. Individual microvessels were counted in four separate fields in this area. Two pathologists, blinded to the protocol, examined all slides in each group on three separate occasions. Data were expressed as the average number of four fields that were counted.

Statistical analysis

Data are expressed as mean ± SE. Statistical analysis was performed using Student's unpaired t-test. $P < 0.05$ was considere statistically significant.

RESULTS

Expressions of PPARγ and RXRα in human pancreatic carcinoma cell line PANC-1

RT-PCR showed that PPARγ mRNA and RXRα mRNA were expressed in PANC-1 cells (Figure 1). Immunocytochemical staining showed that PPARγ and RXRα protein were also expressed in PANC-1 cells (Figure 2). These results indicate that PANC-1 cell line significantly expressed PPARγ and RXRα, and could be used in this protocol.

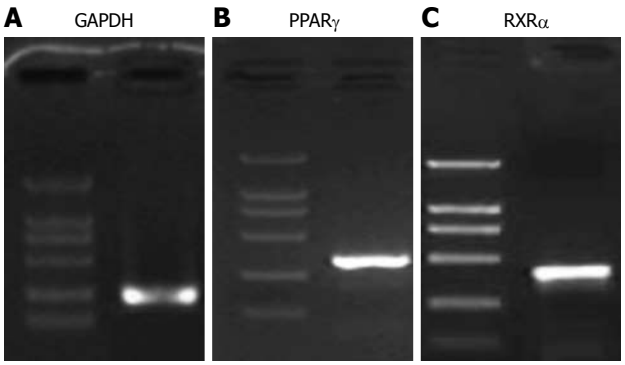

Figure 1 RT-PCR showing expression of 341 bp PPARγ (A) and 422 bp RXRα mRNA (B) in PANC-1 cells. Three micrograms of total RNA from PANC-1 cell line was subjected to RT-PCR. The PCR products were electrophoresed on 1.5% agarose gel. The products were visualized with ethidium bromide staining. GAPDH was used as an internal control. PPARγ, RXRα and GAPDH were detected at the expected molecules sizes.

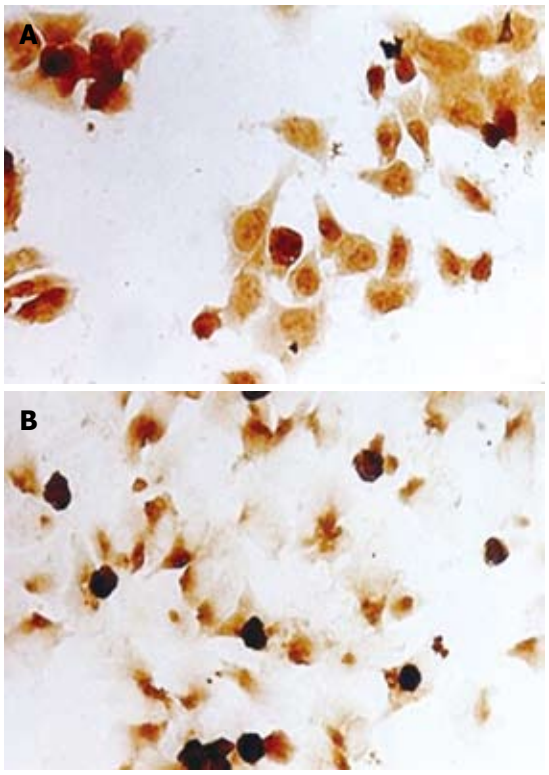

Figure 2 Immuncytochemistry study for the expressions of PPARγ (A) and RXRα protein (B) in PANC-1 cells. Cells were cultured in 12-well plates containing slides. Slides were fixed, washed, and incubated with mouse anti-PPARγ (× 150 dilution) or rabbit anti-RXRα antibody (× 100 dilution), then incubated with the secondary antibody labeled with HRP. The staining was detected using DAB and demonstrated expressions of the nuclei with brown staining (HE staining, × 200).

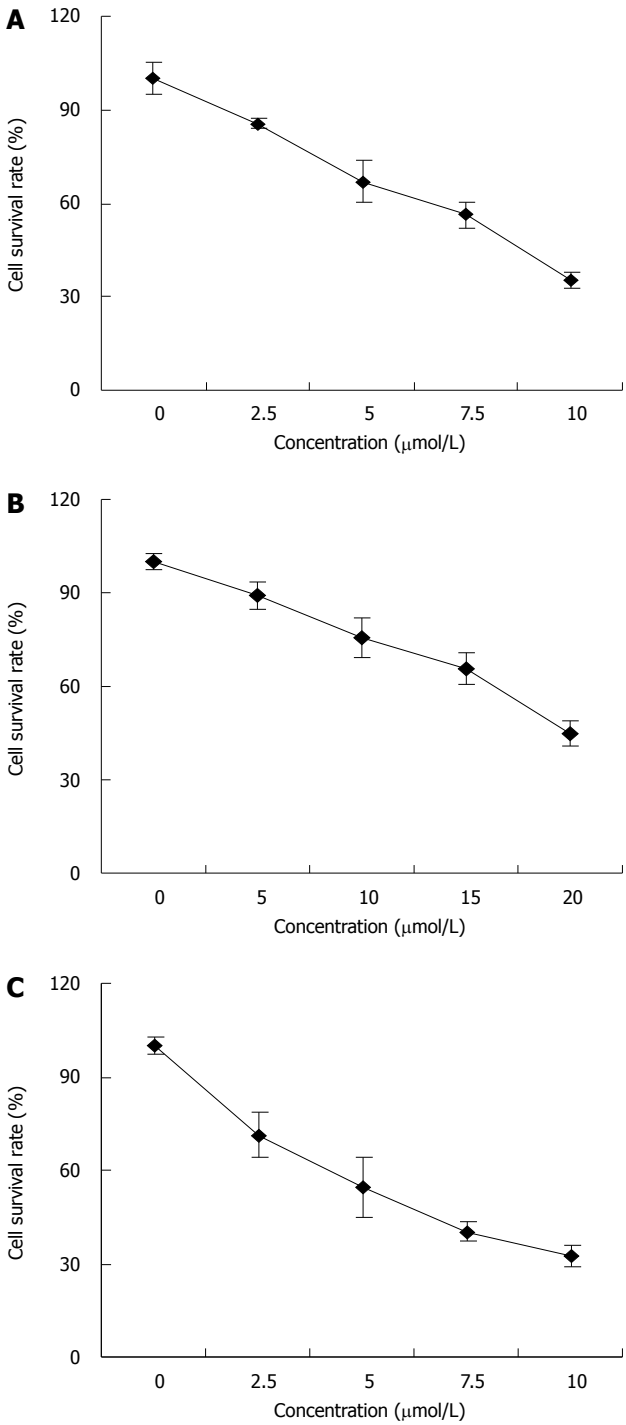

Figure 3 Dose-dependent effects of PPARγ and RXRα ligands on the growth of human pancreatic carcinoma in 15d-PGJ2 group (A), 9-cis-RA group (B), and combined 15d-PGJ2 and 9-cis-RA group (C). Cells were seeded into 96-well plates and treated with different concentrations of 15d-PGJ2, 9-cis-RA and their combination. MTT was used to determine the cellular proliferation. Asterisks indicate statistically significant differences ($P < 0.05$, $n = 6$) based on a two-tailed Student's t-test. Experiments were performed in triplicate.

Effects of 15d-PGJ$_2$ and 9-cis-RA on proliferation of PANC-1 cell line

MTT assay demonstrated that 15d-PGJ$_2$, 9-cis-RA and their combination inhibited the growth of PANC-1 cells in a dose-dependent manner. PANC-1 cells were suppressed to more than 50% of control group at the concentration of 10 μmol/L 15d-PGJ$_2$, 20 μmol/L 9-cis-RA and 5 μmol/L 15d-PGJ$_2$ plus 10 μmol/L 9-cis-RA, respectively. 9-cis-RA had a combined action with 15d-PGJ$_2$ on the growth of pancreatic carcinoma (Figure 3).

Effects of 15d-PGJ$_2$ and 9-cis-RA on expression of VEGF in PANC-1 cells

VEGF is a major stimulator of tumor angiogenesis. We used semi-quantitative RT-PCR to determine whether

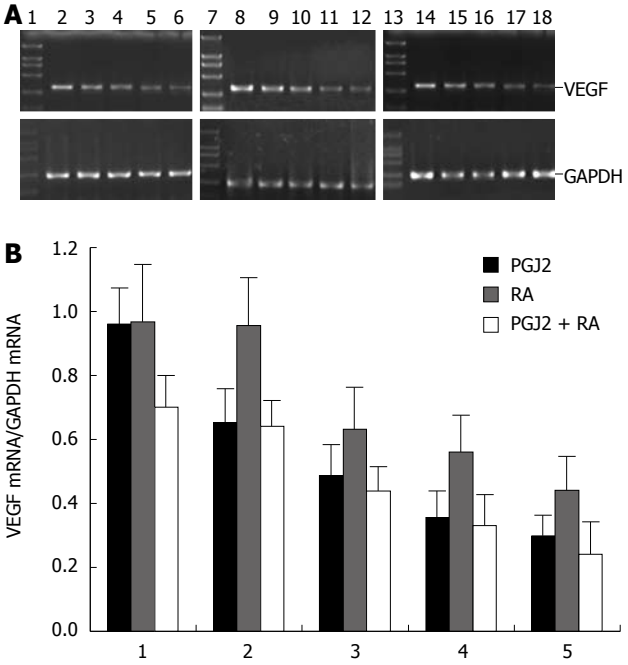

Figure 4 Dose-dependent inhibitory effects of 15d-PGJ2 and 9-cis-RA on expression of VEGF. A: Three micrograms of total RNA from PANC-1 cell line was subjected to RT-PCR. The PCR products were electrophoresed on 1.5% agarose gel and visualized with ethidium bromide staining. GAPDH was used as a control. VEGF expression was detected at the expected molecules sizes (382 bp). Lanes 1, 7, 13: size marker; lanes 2-6, cells treated with 15d-PGJ2 at 0, 2.5, 5, 7.5, 10 μmol/L, respectively; lanes 8-12, cells treated with 9-cis-RA at 0, 5, 10, 15, 20 μmol/L, respectively; lanes 14-18, cells treated with combined 9-cis-RA (10 μmol/L) and 15d-PGJ2 at 0, 2.5, 5, 7.5, 10 μmol/L, respectively; B: Semi-quantitative analysis of VEGF mRNA expression in human pancreatic carcinoma PANC-1 cell line. Data on PCR products were obtained by comparing the intensity of PCR band of VEGF with that of internal control of GAPDH using genetools software.

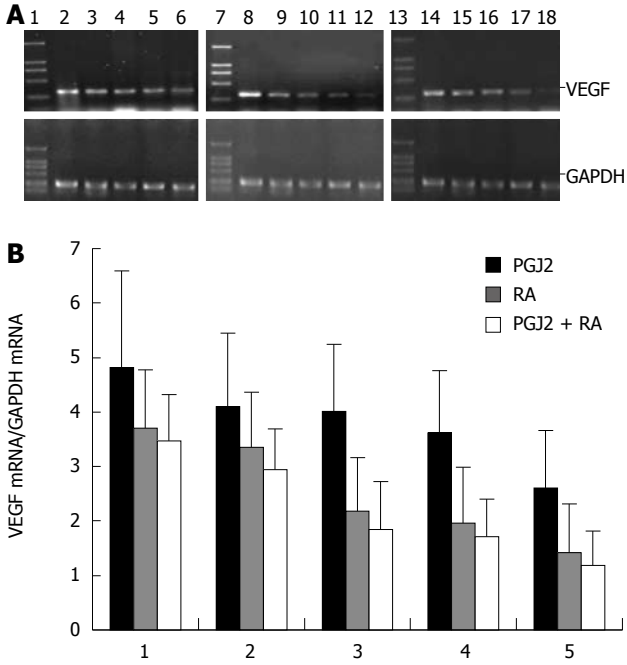

Figure 5 Time-dependent inhibitory effects of 15d-PGJ2 and 9-cis-RA on expression of VEGF mRNA. A: Three microgram of total RNA from PANC-1 cell line was subjected to RT-PCR. The PCR products were electrophoresed on 1.5% agarose gel and visualized with ethidium bromide staining. GAPDH was used as a control. VEGF expression was detected at the expected molecules sizes (382 bp). Lanes 1, 7, 13: size marker; lanes 2-6: cells treated with 10 μmol/L 15d-PGJ2 for 0, 12, 24, 48, 72 h, respectively; lanes 8-12: cells treated with 20 μmol/L 9-cis-RA for 0, 12, 24, 48, 72 h, respectively; lanes 14-18: cells treated with combined 10 μmol/L 9-cis-RA and 5 μmol/L 15d-PGJ2 for 0, 12, 24, 48, 72 h, respectively; B: Semi-quantitative analysis of VEGF mRNA expression in human pancreatic carcinoma PANC-1 cell line. Data on PCR products were obtained by comparing the intensity of PCR band of VEGF with that of internal control of GAPDH using genetools software.

15d-PGJ$_2$ and 9-cis-RA exert any effects on the expression of VEGF mRNA. RT-PCR demonstrated that 15d-PGJ$_2$, 9-cis-RA and their combination inhibited the expression of VEGF mRNA in PANC-1 cells in a dose- and time-dependent manner (Figures 4 and 5).

Effect of Rosiglitazone on PANC-1 cell tumor growth in nude mice

As shown in Figure 6, over a 75-d experimental period, Rosiglitazone (10 μmol/kg. d) inhibited the PANC-1 cell tumor growth. Existing tumors began to regress 3 wk after treatment. The average tumor size was (0.48 ± 0.23) cm^3 in Rosiglitazone treatment group and (2.488 ± 0.59) cm^3 in control group ($P < 0.05$, $n = 15$). The inhibitory rate was 80.7%. The average weight was (0.887 ± 0.48) g in Rosiglitazone treatment group and (2.333 ± 1.66) g in control group ($P < 0.05$, $n = 15$). Tumors in 2 of 15 Rosiglitazone-treated mice were not palpable, indicating a complete regression of these tumors after drug administration. Hepatic function analysis demonstrated that no drug-related hepatic toxicity to any of the mice (Table 1).

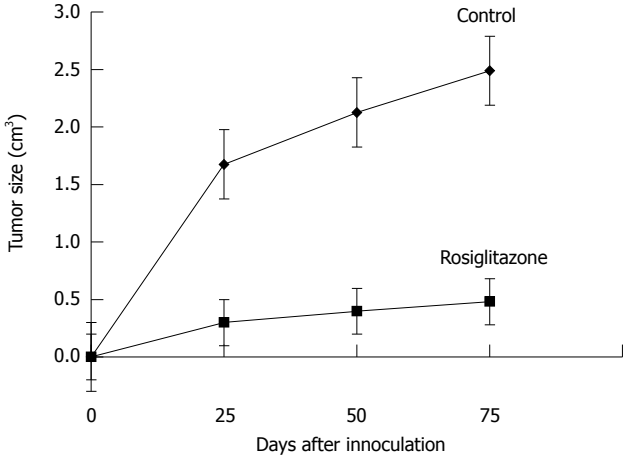

Figure 6 Rosiglitazone supresses PANC-1 cell tumor growth in nude mice. From the second week of PANC-1 cell inoculation, tumor bearing mice were randomized to receive either distilled water or Rosiglitazone, 10 μmol/kg a day, for 75 d. Tumor size was measured every 25 d. Tumor size in Rosiglitazone treatment group was significantly smaller than that in control group ($P < 0.05$).

Effect of Rosiglitazone on expression of PCNA in xenograft tumor of nude mice

Immunohistochemical study revealed that Rosiglitazone had a substantial effect on tumor cell proliferation as detected with staining of antibody against PCNA (Figure 7). PCNA was presented in both groups. PCNA was obviously down-regulated in Rosiglitazone treatment group compared with control group.

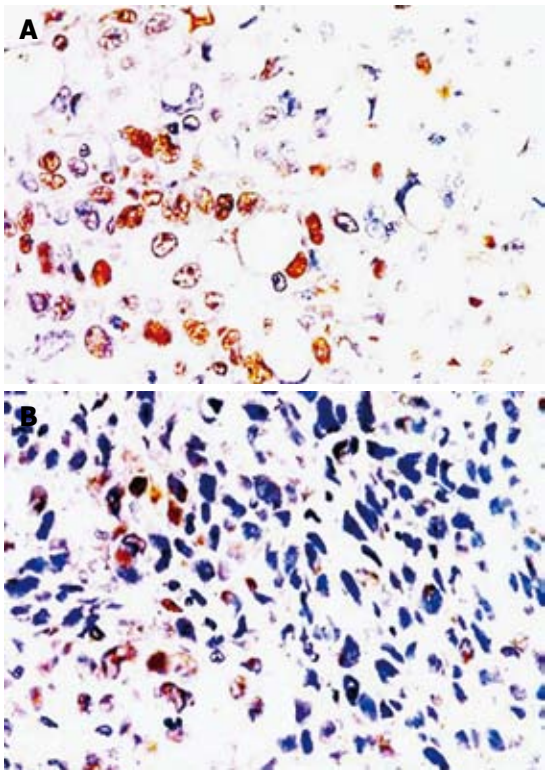

Figure 7 Immunohistochemical analysis of PCNA expression in tumor tissue from control group (A) and Rosiglitazone treatment group (B). From the second week of PANC-1 cell inoculation, tumor bearing mice were randomized to receive either distilled water or Rosiglitazone, 10 μmol/kg per day, for 75 d. Mice were then euthanized. Tumors were removed and cut into 4-μm thick sections for immunohistochemical analysis. The sections were incubated with anti-PCNA antibody, and then with secondary antibody labeled with HRP. The staining was detected using DAB and demonstrated as expression of nuclei with brown staining. The result showed that PCNA expression was significantly down regulated in Rosiglitazone treatment group (original magnification × 200).

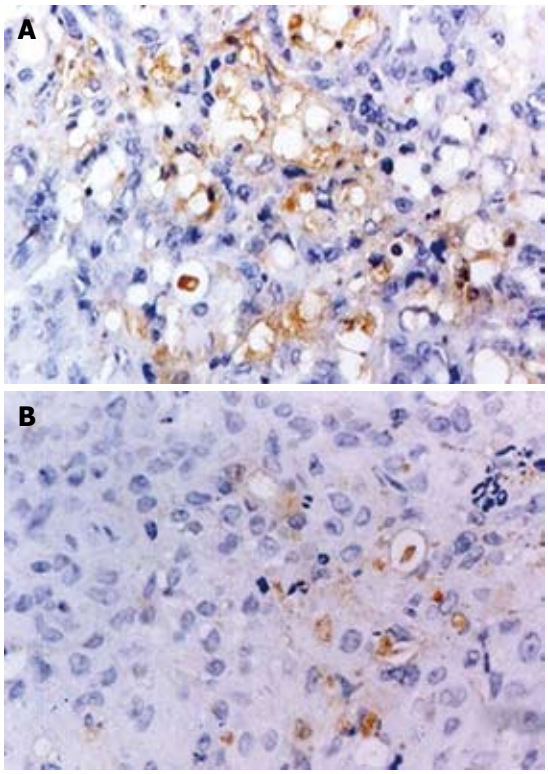

Figure 8 Immunohistochemical analysis of microvessel density in tumor tissue from control group (A) and Rosiglitazone treatment group (B). From the second week of PANC-1 cell inoculation, tumor bearing mice were randomized to receive either distilled water or Rosiglitazone, 10 μmol/kg per day for 75 d. Mice were then euthanized. Tumors were removed and cut into 4 μm-thick sections for immunohistochemical analysis. The sections were incubated with anti-Ⅳ collagen antibody, and then with secondary antibody labeled with HRP. The staining was detected using DAB. A single microvessel was defined as any immunohistochemistry- stained brown endothelial cell separated from the adjacent microvessels, tumor cells, and other connective tissue elements. MVD was calculated. The result showed that MVD was significantly down regulated in Rosiglitazone treatment group. (original magnification × 200).

Table 1 Alterations of liver function analyses in two groups (mean ± SE)

	Rosiglitazone treatment	Control	*P* value
AST	123.6 ± 17.9	132.1 ± 22.6	0.51
ALT	23.2 ± 7.3	21.4 ± 9.6	0.74
LDH	1953.8 ± 324.5	2037.7 ± 378.4	0.70
AMYL	86.5 ± 24.1	73.5 ± 22.1	0.33

Effect of Rosiglitazone on angiogenesis of xenograft tumor in nude mice

Staining with anti-Ⅳ collagen antibody demonstrated that MVD was significantly decreased in Rosiglitazone treatment group, which was (10.67 ± 3.07) in Rosiglitazone treatment group and (31.44 ± 6.06) in control group ($P < 0.01$, $n = 30$). Tumor blood vessels in control group showed a sinusoidal pattern and rather well-developed vascular networks. In contrast, tumor blood vessels in Rosiglitazone treatment group were consisted of randomly distributed endothelial cells that did not form organized vascular networks (Figure 8).

Effect of Rosiglitazone on VEGF expression in xenograft tumor of nude mice

To determine whether Rosiglitazone exerts its inhibitory effect on tumor angiogenesis by suppressing VEGF, tumor tissues from mice in control and Rosiglitazone treatment groups were assayed for the expression of VEGF mRNA. Semi-quantitative RT-PCR showed that the expression of VEGF mRNA was down-regulated in Rosiglitazone treatment and control groups (Figure 9).

DISCUSSION

PPAR, a member of the family of ligand-activated nuclear receptor transcription factors, forms a heterodimer with the RXR upon ligand binding. This complex binds to a PPAR-responsive element (PPRE) located in the promoter region of various genes and acts to regulate their expression. There are three members of the PPAR family, α, β and γ. PPARα is highly expressed in the liver, heart, proximal tubules of kidney, and intestinal mucosa. PPARβ is almost ubiquitously expressed in adipose tissue, and PPARγ is highly expressed in adipose tissue where it plays a critical role in the differentiation of preadipocytes into adipocytes. High concentrations of PPARγ protein have also been identified in the colon. PPARγ is also expressed in the immune system including the spleen, monocytes, and bone-marrow precursors. Recently, great attention

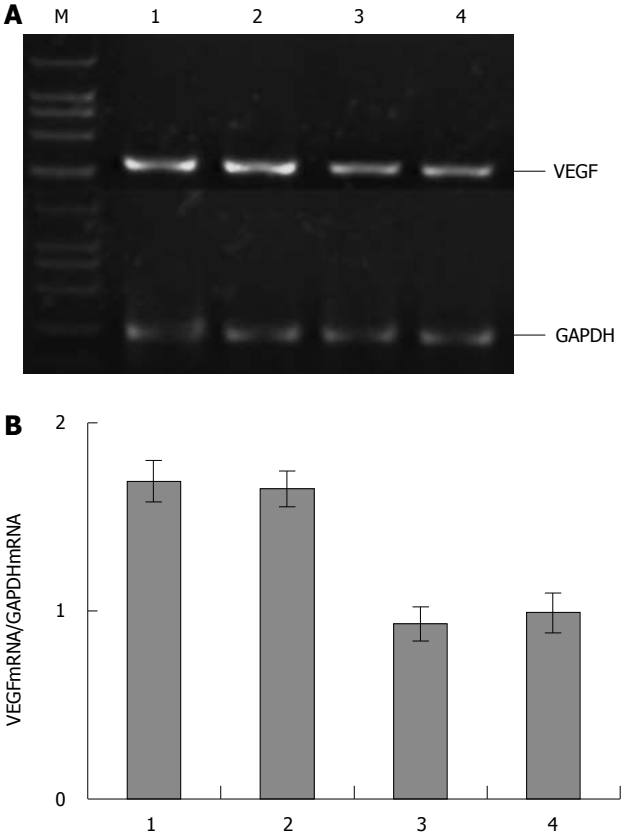

Figure 9 RT-PCR showing expression of VEGF mRNA in human pancreatic carcinoma PANC-1 cell xenograft tumor. A: Three micrograms of total RNA from PANC-1 cell xenograft tumor tissue was subjected to RT-PCR. The PCR products were electrophoresed on 1.5% agarose gel. GAPDH was used as a control. VEGF expression was detected at the expected molecule sizes of 382 bp (M: size marker; lanes 1-2: control mice; lanes 3-4: Rosiglitazone-treated mice); B: Semi-quantitative analysis of VEGF mRNA expression in human pancreatic carcinoma PANC-1 cell xenograft tumor tissue. Data on PCR products were obtained by comparing the intensity of the PCR band of VEGF with that of internal control of GAPDH using genetools software. The result showed that expression of VEGF mRNA in tumor tissue was down-regulated in Rosiglitazone treatment group compared with control group.

has focused on PPARγ. PPARγ plays an important role in insulin sensitization and differentiation of adipocytes, monocytes and macrophages[5]. Activation of PPARγ can also induce growth inhibition in human prostate cancer cells, colon cancer cells, gastric cancer cells[10], pancreatic cancer cells[12-17] and liposarcoma cells[18].

The expression of PPARγ and its role in human pancreatic carcinoma have not been fully elucidated. We have previously reported that PPARγ is the only isoform of PPAR which exists in PC-3 pancreatic carcinoma cell line[20]. In this study, PPARγ and RXRα were strongly expressed in human pancreatic carcinoma cell line PANC-1, suggesting that PPARγ might play a role in the regulation of pancreatic cancer growth. In addition, we demonstrated an antiproliferative effect of PPARγ and RXRα ligands on pancreatic cancer growth pancreatic cancer growth *in vitro*. MTT assay demonstrated that 15d-PGJ$_2$, 9-cis-RA and their combination inhibited the growth of PANC-1 cells in a dose-dependent manner. PANC-1 cells were suppressed to more than 50% of the control group at 10 μmol/L of 15d-PGJ$_2$ and 20 μmol/L of 9-cis-RA. The inhibitory effect of 5 μmol/L 15d-PGJ$_2$ plus 10 μmol/L 9-cis-RA on cancer cell growth was stronger than that of 5 μmol/L 15d-PGJ$_2$ or 10 μmol/L 9-cis-RA alone. These results indicate that PPARγ ligand, 15d-PGJ$_2$, and RXRα, 9-cis-RA, can strongly inhibit the growth of pancreatic cells *in vitro*, and 9-cis-RA combined with 15d-PGJ$_2$ can inhibit the growth of pancreatic carcinoma, further confirming that activation of PPARγ and RXRα may suppress pancreatic carcinoma growth *in vitro*[15]. To investigate the *in vivo* effect of PPARγ ligand, a pancreatic carcinoma xenograft model of nude mice was established by inoculating PANC-1 cells subcutaneously, and Rosiglitazone, a PPARγ activator, was administered *via* water drinking in experimental group for 75 d. Systemic administration of Rosiglitazone not only inhibited PANC-1 tumor cell growth, but also dramatically reduced the size of existing tumors. The average tumor volume and weight in experimental group were less than those in the control group. The growth inhibition rate of Rosiglitazone was 80.7%. In addition, PCNA, as an important marker of cell proliferation, was presented in both groups, but immunohistochemistry showed that PCNA was down regulated in experimental group compared with the control group. However, such a dosage of Rosiglitazone had no systemic toxic action. Take these into account, activation of PPARγ could inhibit pancreatic cancer growth.

Although the mechanism of PPARγ by which the growth of human pancreatic carcinoma cells is inhibited has not been fully elucidated, its action on inducing apoptosis and G1 cell cycle arrest has been reported[15]. As other tumors, pancreatic carcinoma is also neovascularization dependent. This process is usually mediated by angiogenic factors. Of these factors, VEGF is currently the major proangiogenic factor for most types of human cancer including pancreatic carcinoma[21,22]. In our study, 15d-PGJ$_2$, 9-cis-RA and their combination inhibited the expression of VEGF in PANC-1 cells in a dose- and time-dependent manner. *In vivo* study showed that the expression of VEGF in tumor tissue was significantly down-regulated in Rosiglitazone treatment group compared with control group. Furthermore, Rosiglitazone can also reduce pancreatic carcinoma microvessel density, a marker of tumor angiogenesis, indicating that PPARγ ligand can inhibit cancer growth *in vitro* or *in vivo*, which might be, at least in part, related to the suppression of tumor angiogenesis. VEGF may be one of the important factors related to the suppression of angiogenesis in pancreatic carcinoma by activating PPARγ.

In summary, human pancreatic carcinoma cell line PANC-1 expresses PPARγ, and activation of PPARγ inhibits cellular growth. Suppression of tumor angiogenesis by down-regulating the expression of VEGF may be one of the mechanisms of PPARγ activation by which pancreatic carcinoma growth is inhibited, thus providing a new insight into the mechanism of PPARγ ligand, as an inhibitor of pancreatic tumor growth and a novel means to control pancreatic carcinoma in clinical practice.

COMMENTS

Background

Peroxisome proliferator-activated receptor γ (PPARγ), a member of the family

of ligand-activated nuclear receptor transcription factors, is expressed in many human solid tumors. It was reported that activation of PPARγ can inhibit the growth of pancreatic carcinoma cells, colon cancer cells, gastric cancer cells and liposarcoma cells. So far, the underlying mechanism of PPARγ activation by which the growth of human pancreatic carcinoma is inhibited has not been fully elucidated.

Research frontiers

The growth and progression of solid tumors including pancreatic carcinoma depend on angiogenesis. Vascular endothelial growth factor (VEGF) is the major proangiogenic factor for most types of human cancer. However, whether activation of PPARγ is related with expression of VEGF in human pancreatic carcinoma is still unclear.

Innovations and breakthroughs

Human pancreatic carcinoma cell line PANC-1 expresses PPARγ, and activation of PPARγ inhibits cellular growth both *in vivo* and *in vitro*. Pancreatic carcinoma microvessel density, a marker of tumor angiogenesis, was reduced in this study. Moreover, suppression of tumor angiogenesis by down-regulating the expression of VEGF may be one of the mechanisms of PPARγ activation by which the growth of pancreatic carcinoma is inhibited, thus providing a new insight into the mechanism of PPARγ ligand, as an inhibitor of pancreatic tumor growth.

Applications

Suppression of tumor angiogenesis by down-regulating the expression of VEGF may be one of the mechanisms of PPARγ activation by which the growth of pancreatic carcinoma is inhibited, suggesting that the drugs used in our study can control pancreatic carcinoma in clinical practice.

Terminology

PPAR is a member of the family of ligand-activated nuclear receptor transcription factors and forms a heterodimer with the RXR upon ligand binding. This complex then binds to a PPAR-responsive element (PPRE) located in the promoter region of various genes and acts to regulate their expression.

Peer review

This is a well designed study. The results suggest that activation of peroxisome PPARγ suppresses pancreatic carcinoma growth by inhibiting angiogenesis.

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Capecitabine and irinotecan with and without bevacizumab for advanced colorectal cancer patients

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Abstract

AIM: To investigate the efficacy and safety of capecitabine plus irinotecan ± bevacizumab in advanced or metastatic colorectal cancer patients.

METHODS: Forty six patients with previously untreated, locally-advanced or metastatic colorectal cancer (mCRC) were recruited between 2001-2006 in a prospective open-label phase Ⅱ trial, in German community-based outpatient clinics. Patients received a standard capecitabine plus irinotecan (CAPIRI) or CAPIRI plus bevacizumab (CAPIRI-BEV) regimen every 3 wk. Dose reductions were mandatory from the first cycle in cases of > grade 2 toxicity. The treatment choice of bevacizumab was at the discretion of the physician. The primary endpoints were response and toxicity and secondary endpoints included progression-free survival and overall survival.

RESULTS: In the CAPIRI group *vs* the CAPRI-Bev group there were more female than male patients (47% *vs* 24%), and more patients had colon as the primary tumor site (58.8% *vs* 48.2%) with fewer patients having sigmoid colon as primary tumor site (5.9% *vs* 20.7%). Grade 3/4 toxicity was higher with CAPIRI than CAPIRI-Bev: 82% *vs* 58.6%. Partial response rates were 29.4% and 34.5%, and tumor control rates were 70.6% and 75.9%, respectively. No complete responses were observed. The median progression-free survival was 11.4 mo and 12.8 mo for CAPIRI and CAPIRI-Bev, respectively. The median overall survival for CAPIRI was 15 mo (458 d) and for CAPIRI-Bev 24 mo (733 d). These differences were not statistically different. In the CAPIRI-Bev, group, two patients underwent a full secondary tumor resection after treatment, whereas in the CAPIRI group no cases underwent this procedure.

CONCLUSION: Both regimens were well tolerated and offered effective tumor growth control in this outpatient setting. Severe gastrointestinal toxicities and thromboembolic events were rare and if observed were never fatal.

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Key words: First-line therapy; Metastatic colorectal cancer; Bevacizumab; Capecitabine; Irinotecan; Tumor response

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INTRODUCTION

Until relatively recently, the anti-metabolite 5-fluorouracil (5-FU) was the only effective first-line treatment for metastatic colorectal cancer (mCRC). Subsequent attempts to improve its efficacy centered on its co-administration with leucovorin (LV)[1]. An alternative approach to optimize 5-FU-based therapy was made possible with the advent of oral fluoropyrimidine derivatives. Capecitabine is such an oral FU pro-drug specifically designed to deliver 5-FU to tumor cells with predictable bioavailability and sustained exposure[2-4]. Capecitabine has shown promising results and clinically meaningful safety advantages over 5-FU/LV, although with no improved efficacy[5-8]. Its oral administration makes it more convenient, and its proven safety might increase its appeal to patients who have expressed a preference for oral cytotoxic therapy over intravenous regimens (iv)[9,10].

Additionally, over the last decade improved survival has been achieved with the arrival of new agents, including irinotecan and oxaliplatin[11,12]. Two large randomized clinical trials have shown that irinotecan combined with 5-FU/LV (FOLFIRI or bolus IFL) significantly improved response rates, time to progression and overall survival[12,13]. Based on data from these, and other trials[12-16] the combination regimens FOLFIRI and oxaliplatin/5-FU/LV (FOLFOX) replaced 5-FU/LV as a standard treatment of mCRC.

Thus, following promising data from phase Ⅰ/Ⅱ trials in colorectal cancer (CRC), interest in the combination of capecitabine with irinotecan (CAPIRI) as an equally effective, more convenient alternative to the FOLFIRI and FOLFOX regimens has grown[17-19]. CAPIRI in the first-line treatment of advanced disease has been associated with response rates of 38%-45% and a time-to-progression (TTP) of about 8 mo with manageable side-effect profiles[20-24], although in some studies an acceptable safety was only achieved by lowering the irinotecan doses[21-23].

Furthermore, the most recent advances in the treatment of mCRC have been possible with the advent of targeted drugs. These include bevacizumab, a recombinant humanized monoclonal antibody to the vascular endothelial growth factor VEGF-A; the predominant member of the VEGF ligand family which is mainly involved in tumor angiogenesis[25]. Bevacizumab was approved by the European Medicines Agency in 2004 for use in combination with any i.v. 5-FU-based therapy for CRC in the first-line setting[26]. Although the trials could be criticized in the choice of a bolus 5-FU/LV regimen which is now considered to be obsolete, a consistent benefit from the addition of bevacizumab has been demonstrated in several studies, including those with capecitabine[27-33].

Whilst still under debate, it appears that combinations of capecitabine, irinotecan and bevacizumab may improve outcome of mCRC patients in trial-experienced hospital centers. However, as many small non-university community-based outpatient clinics are slow to adapt to new combination protocols due to their lack of experience in clinical studies and unfamiliarity with the side effects of these novel combinations, patient data from these out-patient clinics may be quite different to that usually presented from large trials. In the absence of available data on safety and efficacy of CAPIRI +/- bevacizumab (CAPIRI-Bev) in community-based out-patient clinics, we initiated this prospective open-label multicentric trial with a high emphasis placed on such typically community-based out-patient clinics not participating in other clinical mCRC trials[15,16].

MATERIALS AND METHODS

Patient eligibility, study design and treatment

Patients with previously untreated locally advanced or mCRC were eligible for entry into this trial. Histological confirmation of CRC or CRC liver metastases that were not resectable at the time of patient inclusion in the study was required. Additional requirements were an Eastern Cooperative Oncology Group (ECOG) performance status of ≤ 2 (Karnofsky index > 70%) at least one measurable lesion and normal major organ function. Patients were eligible for inclusion, if a previous adjuvant chemotherapy regimen did not include irinotecan and/or bevacizumab. Patients did not have any age restrictions, but life expectancy had to be at least 3 mo.

This was a prospective, open-label multicentric non randomized phase Ⅱ study conducted by Mainz University, with an inclusion period from 2001 to 2006, particularly in nine small community-based German out-patient clinics. Patients were consecutively recruited according to whether they had received either a standard CAPIRI-regimen [capecitabine 1000 mg/m² twice daily (bid), on days 1-14 and irinotecan 200 mg/m² on day 1] or the same regime CAPIRI combined with bevacizumab (CAPIRI-Bev plus bevacizumab 7.5 mg/kg body weight, also on day 1). Patients in both groups received treatment, every 3 wk, in regional out-patient clinics in Germany; treatment choice of bevacizumab was at the discretion of the physician. Treatment continued until first documented tumor progression under therapy. Treatment was halted in cases of clinical progression, severe therapy-associated adverse events and withdrawal of written informed consent. The protocol was approved by the local ethics review boards of all participating institutions. Patient follow up commenced with treatment initiation and ended on 25th July 2008 or with the death of a patient. The resulting median follow-up times for CAPIRI and CAPIRI-Bev were similar, 19.5 and 17.0 mo, respectively.

Participation in the study required patient written informed consent. Patients who had undergone a major surgical procedure within 4 wk before start of treatment were excluded from the trial. Major exclusion criteria included previous treatment with capecitabine, irinotecan and bevacizumab in a palliative setting and participation in another study during treatment. Patients with malignant tumors other than basalioma, successfully treated in situ carcinoma of the cervix or a tumor relapse free period more than 5 years, were excluded from the trial.

Due to the toxicities associated with bevacizumab,

patients with myocardial infarction within 1 year before start of treatment, stroke, thromboembolic events, severe bleeding within 6 mo prior to treatment, hemorrhagic diathesis, unhealing wounds or fractures or proteinuria $\geqslant 1$ in urine sample were excluded. Anticoagulant treatment, thrombocyte-aggregation inhibitors, as well as St. John's wort were prohibited on the date of enrollment to our study.

Statistical analysis

Endpoints: Primary endpoints were response rates (RR) and the toxicity profile; secondary endpoints were progression free survival (PFS) and overall survival (OS) under study treatment.

Response assessment: Tumor response classification was based on the definitions set out in the Response Evaluation Criteria in Solid Tumors (RECIST)[34]. Tumor response was assessed after 12 wk (4 cycles) and all responses were confirmed by further radiological assessment every 12 wk if applicable. The following parameters were calculated: response rates, PFS and OS. The tumor control rate was defined as: complete response (CR) + partial response (PR) + stable disease (SD).

Toxicity measurements: Toxicity was defined as the frequency of hematological and non-hematological, chemotherapy associated adverse events and laboratory changes after 3 mo of treatment. Toxicity of treatment was evaluated at all visits and graded 1 to 4 using the National Cancer Institute Common Toxicity Criteria (NCI-CTC) Version 2.0. For each toxic event the documented maximal NCI-CTC grading throughout treatment was taken into account, to record chemotherapy-associated toxicity in both treatment groups. Particular emphasis was placed on the management of late-onset diarrhea.

Statistical analysis: The study was not set up to significantly compare both groups. However statistical analysis was performed using the full analysis set (intent-to-treat). Statistical analysis including survival analysis according to Kaplan-Meier was performed with the SPSS software package. Survival was measured from the time of diagnosis to the date of death or last follow-up. PFS were calculated from the first day of treatment until progression. Differences in the toxicity profile of both study groups were analyzed by χ^2 test. Value of significance was calculated *via* Fisher's test and $P < 0.05$ was considered significant.

RESULTS

Patient population

A total of 46 patients were enrolled and included in the intent-to-treat analysis of this prospective trial, of whom 17 (37.0%) received treatment with CAPIRI and 29 (63.0%) treatment with CAPIRI-Bev. The patient demographics and baseline characteristics are presented in Table 1. Since patients were not directly randomized but included into the treatment groups at the discretion of the investigators, some clinical parameters (e.g. gender, location of rectum, lymph node metastasis) differed between both groups, but were not statistically different. Patients' age was similar between both patient groups, however there were more female patients in CAPIRI than in the CAPIRI-Bev group (47.1% *vs* 24.1%). The colon was more frequently the primary tumor site in CAPIRI than in the CAPIRI-Bev group (58.8% *vs* 48.2%), which contained fewer patients with sigmoid colon as primary site (5.9% *vs* 20.7%). The liver was the most common metastatic site in both groups (71% and 83%), followed by lymphatic and pulmonary metastases. Previous adjuvant chemotherapy regimen consisted of 5-FU/LV for 6 patients receiving CAPIRI and 4 patients receiving CAPIRI-Bev, and FOLFOX for 2 patients receiving CAPIRI and 4 receiving CAPIRI-Bev.

Table 1 Patient characteristics

	Treatment group	
	CAPIRI	CAPIRI-Bev
N (%)	17 (37%)	29 (63%)
Age (median), years (range)	66 (55-81)	60 (37-80)
Gender, M/F	9/8	22/7
Site of primary tumor, *n* (%)		
Rectum	7 (41.2)	15 (51.7)
Colon	10 (58.8)	14 (48.2)
Site of metastases, *n* (%)[1]		
Hepatic	12 (71)	24 (83)
Pulmonary	6 (35)	11 (38)
Lymphatic and nodal	4 (24)	17 (59)
Skeletal	1 (6)	4 (14)
Peritoneal	1 (6)	5 (17)
Other sites	6 (35)	5 (17)
Performance status (Karnofsky)		
100%	4 (23.5)	17 (58.6)
90%	1 (5.9)	5 (17.2)
85%	1 (5.9)	0 (0)
80%	2 (11.8)	3 (10.3)
70%	4 (23.5)	0 (0)
Adjuvant chemotherapy received	8 (47%)	8 (27.6%)

[1]Patients could have more than one metastatic site.

Treatment compliance

The median number of cycles received was 9 ($n = 17$, CAPIRI) and 8 ($n = 29$, CAPIRI-Bev), and the median duration of therapy was 133 d (> 4 mo) for both groups. Patients in the CAPIRI group received a median dose of 3500 mg capecitabine and 400 mg irinotecan per cycle whereas patients in the CAPIRI-Bev group received 3876 mg capecitabine and 382 mg irinotecan per cycle. At least 1 dose reduction had to be undertaken in 9/29 patients (31%) treated with CAPIRI-Bev and 8/17 patients (47%) treated with CAPIRI. In 55% of the CAPIRI and 53% of the CAPIRI-Bev patients, treatment had to be delayed at least once. The number of hospital admissions during treatment with CAPIRI-Bev was higher than during treatment with CAPIRI (44.8% *vs* 29.4%).

Toxicity

The incidence of hematological and non-hematological

Table 2 Incidence of chemotherapy-related toxicities

	Treatment group			
	CAPIRI (n = 17)		CAPIRI-Bev (n = 29)	
NCI-CTC Grade	Total (1-4)	3-4	Total (1-4)	3-4
Hematologic toxicities, number (%) patients				
Anemia	2 (11.8)	1 (5.9)	4 (13.8)	-
Leukopenia	4 (23.5)	2 (11.8)	6 (20.7)	1 (3.4)
Neutropenia	3 (17.6)	1 (5.9)	5 (17.2)	2 (6.9)
Non-hematologic toxicities, number (%) patients				
Diarrhea	7 (41.2)	5 (29.4)	13 (44.8)	5 (17.2)
Nausea/vomiting	6 (35.3)	-	12 (41.4)	2 (6.9)
Hand-foot syndrome	6 (35.3)	1 (5.9)	9 (31.0)	-
Mucositis	3 (17.3)	2 (11.8)	5 (17.2)	-
Cholinergic syndrome	3 (17.3)	-	2 (6.9)	-
Alopecia[1]	2 (11.8)	-	12 (41.4)	-
Fatigue	2 (11.8)	-	10 (34.5)	-
Anorexia	1 (5.9)	1 (5.9)	2 (6.9)	1 (3.4)
Fever	1 (5.9)	-	4 (13.8)	-
Proteinuria[1]	-	-	7 (24.1)	1 (3.4)
Arterial hypertension	-	-	3 (10.3)	-
Cardiovascular	1 (5.9)	-	1 (3.4)	1 (3.4)
Thromboembolic event	-	-	3 (10.3)	-
GGT rise	1 (5.9)	-	4 (13.8)	4 (13.8)
Hypokalemia	1 (5.9)	1 (5.9)	3 (10.3)	-
Creatinine rise	1 (5.9)	-	1 (3.4)	-

GGT: Gamma-glutamyl transferase; NCI-CTC: National Cancer Institute-Common Toxicity Criteria (version 2), [1]Fisher's test, $P < 0.05$.

toxicities is displayed, by treatment group, in Table 2. Neutropenia and leukopenia were the most frequently reported hematological toxicities, although the number of patients affected by grade 3-4 hematological events was minimal in both groups. Leukopenia was observed in 23.5% *vs* 20.7% (CAPIRI *vs* CAPIRI-Bev). The frequency of neutropenia was equally distributed (17.6% CAPIRI *vs* 17.2% CAPIRI-Bev). Most frequently reported non-hematological toxicities included diarrhea, nausea/vomiting, and hand-foot syndrome (Table 2). The total incidence of diarrhea was similar for CAPIRI and CAPIRI-Bev (41.2% *vs* 44.8%) and grade 3-4 diarrhea was more frequent with CAPIRI (23.5% *vs* 17.2%). Further statistical analysis did not reveal any significant differences between the two groups for non-hematological toxicities. The CAPIRI-Bev group had a significantly higher total incidence of alopecia ($P = 0.049$) and proteinuria ($P = 0.036$), with one Grade 3 proteinuria. Without reaching the level of significance ($P = 0.286$) three cases of arterial hypertension and non-severe thromboembolism were observed for CAPIRI-Bev whereas these events were not observed in the CAPIRI group. All three cases of arterial hypertension were of low NCI-CTC level and were easily managed with oral antihypertensive therapy. One case of minor thromboembolism led to continuation of therapy only without Bev. One cardiovascular event was reported in each group: whereas a stable angina pectoris was reported for CAPIRI, one case of ST-elevated myocardial infarction led to discontinuation of therapy in a patient receiving treatment with CAPIRI-Bev. Thus, the overall grade 3-4 toxicity was reported to be statistically not significant higher in CAPIRI compared to CAPIRI-Bev, 82.4% *vs* 58.6%, respectively.

Table 3 Response rates

	Treatment group	
	CAPIRI (n = 17) number (%) patients	CAPIRI-Bev (n = 29) number (%) patients
Tumor control rate (CR + PR + SD)	13 (70.6)	22 (75.9)
Complete response (CR)	-	-
Partial response (PR)	5 (29.4)	10 (34.5)
Stable disease (SD)	7 (41.2)	12 (41.4)
Progressive disease (PD)	5 (29.4)	6 (20.7)
Not rated	-	1 (3.4)

Table 4 Second-line therapies

	Treatment group	
	CAPIRI (n = 17) number (%) patients	CAPIRI-Bev (n = 29) number (%) patients
Folfiri + Bevacizumab	-	4 (14)
Irinotecan + Cetuximab	-	1 (3)
Capox +/-Bevacizumab	1 (6)	2 (7)
Capox + Cetuximab	-	1 (3)
Folfox +/- Cetuximab	2 (12)	2 (7)
Cetuximab alone	1 (6)	1 (3)
Capiri +/- Cetuximab	-	4 (14)
Capiri + Bevacizumab	1 (6)	-
No second line	10 (59)	14 (48)

Response

Following 3 mo of therapy, response was evaluated in 45 patients. One patient withdrew from CAPIRI-Bev before the 1st assessment. Both groups achieved similar tumor growth control rates (TCR): 70.6% and 75.9% patients treated with CAPIRI or CAPIRI-Bev, respectively. However, there was a difference in response rates, with a not significantly different but higher number of partial responses in the CAPIRI-Bev group (34.5%) compared with the standard CAPIRI group (29.4%) (Table 3). Two patients who achieved a partial response in the CAPIRI-Bev group during the course of treatment were able to undergo a full secondary tumor resection in terms of a curative therapy. No complete responses were observed in either group during follow-up after 3 mo.

Survival

Progression-free survival and overall survival (Figure 1) were in favor of patients receiving CAPIRI-Bev. The median PFS was 11.4 mo (i.e. 342 d) and 12.8 mo (i.e. 385 d) for the CAPIRI or CAPIRI-Bev group, respectively. This difference was not statistically significant (Log Rank, $P = 0.199$). Following second-line protocols in nearly half of all patients (Table 4), the median overall survival was approximately 24 mo in the CAPIRI-Bev group and approximately 15 mo in the CAPIRI group which was not significantly different (Log Rank, $P = 0.53$). The 60 d all

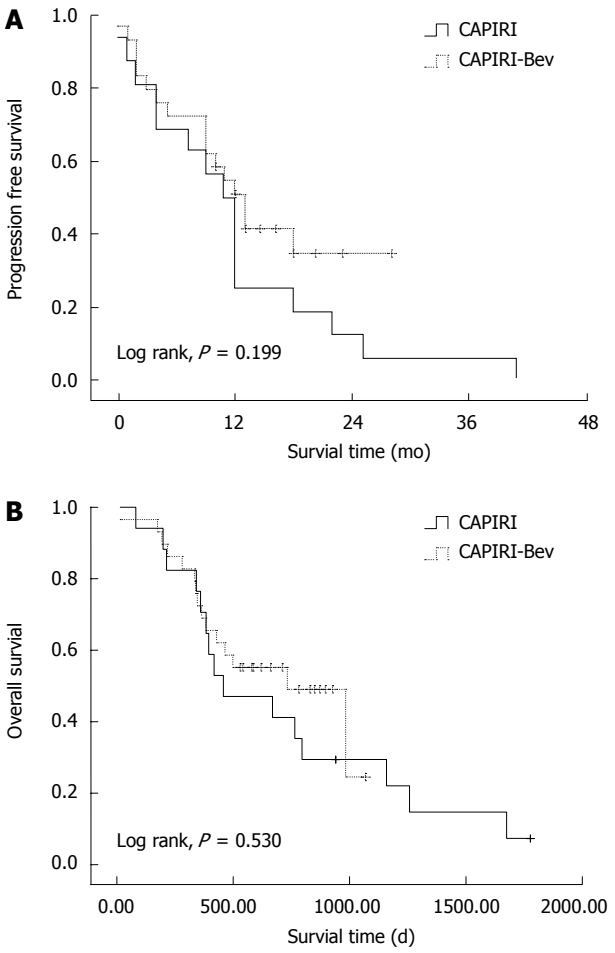

Figure 1 Kaplan Meier survival estimates by treatment group. A: Progression-free survival; B: overall survival. Bev: bevacizumab.

cause mortality was 0% for CAPIRI *vs* 3.4% (1 patient) for CAPIRI-Bev. This patient died 15 d after the first administration of therapy due to unknown cause, (most likely due to progressive disease), with no signs of a cardiovascular or thromboembolic event.

DISCUSSION

This open-label, multicentric, community-based phase Ⅱ trial aimed to prospectively evaluate safety and efficacy of CAPIRI +/- Bev. Most of the 46 patients were recruited by out-patient clinics inexperienced with phase Ⅰ-Ⅲ studies. Thus we propose that these clinics are clearly different from the highly sophisticated centers regularly participating in large registration trials, but however represent the small drug-prescribing centers broadly seen in mCRC patient care for many European and non-European countries. This is also likely to be the reason why the ECOG performance status was not documented in 9 patients. As these primarily conservatively oriented out-patient clinics are relatively slow to adapt to new combination protocols, they also recruited more slowly and enrolled less than one third of their patients into our study (Moehler, personal communications). In addition these patients were those to be treated in a truly palliative setting.

Despite this relatively inexperienced clinical setting, the safety and efficacy of both protocols CAPIRI +/- Bev were remarkably positive. Consistent with other larger trials[29,32,33], a higher RR was obtained with bevacizumab than without (34.5% *vs* 29.4%). The RR in addition to the TCR were almost as high as in a recently presented phase Ⅳ study incorporating FOLFIRI + Bev[35]. Our results also show a higher median PFS and clearly better OS (24 *vs* 15 mo) in patients receiving CAPIRI plus bevacizumab compared to CAPIRI alone, even with less second-line therapies being given (Table 4). Furthermore the increased RR was achieved without a marked increase in toxicity. The incidence of grade 3-4 toxicities was higher in patients not receiving bevacizumab than those receiving it (82.4% *vs* 58.6%, respectively). Notable among these toxicity differences was the higher incidence of Grade 3-4 diarrhea in the CAPIRI group (29.4% *vs* 17.2%), as this was early in recruitment time without clear patient recommendation for the prompt use of loperamide in these relatively inexperienced centers[15,16].

Our efficacy results compare favorably with those obtained with the standard FOLFIRI or FOLFOX regimens[11,12]. Saltz showed in 683 patients, a significant improvement in median PFS and median OS (14.8 *vs* 12.6 mo) and RR were almost doubled; 39% 5-FU/LV + irinotecan *vs* 21% 5-FU/LV, respectively[13]. In the randomized phase Ⅱ study (BICC-C) examining three regimens; FOLFIRI, modified bolus mIFL or CAPIRI, PFS times of 7.8, 6.0 and 5.8 mo and OS of 23.1, 17.6 and 18.9 mo were observed, respectively[36]. After subsequently amending the protocols to include the addition of bevacizumab to the FOLFIRI and mIFL arms, they achieved median PFS times of 11.2 and 8.3 mo and median OS times of 19.2 mo with mIFL + Bev but it was not reached with FOLFIRI + Bev[36].

Furthermore, our results compare well with those from the pivotal phase Ⅲ study with 813 previously untreated patients who received IFL + bevacizumab or placebo[37]. OS for IFL + bev was shown to be 20.3 mo compared to 15.6 mo in the placebo + IFL group. Here, the relatively low median OS for CAPIRI in our study may reflect the fact that our population consisted of patients with poor prognostic factors (5 patients with ECOG PS2) receiving palliative therapy and were not always the typical trial patient population used in large trials. Herein, two large observational studies of the use of chemotherapy plus bevacizumab in broader clinical practice (the BRiTE and BEAT trials) confirm our safety and efficacy results[30,32,33]. The BRiTE community-based registry found that mCRC patients with FOLFIRI + Bev, FOLFOX + Bev, or capecitabine + oxaliplatin (XELOX)-Bev achieved median PFS times of 10.9, 10.3 and 9.0 mo, respectively[30]. The efficacy data from the BEAT community-based trial showed similar median PFS data[32,33].

Thus, in concordance with the early promise of this novel combination in phase Ⅰ/Ⅱ trials[17,18], we postulate that CAPIRI is at least as effective, but is a more convenient alternative to the standard FOLFIRI and FOLFOX regimens. The dose-limiting toxicities were neutropenia

and diarrhea, and dose regimens of capecitabine 1000 mg/m^2 bid for 14 d, and irinotecan of between 250 and 300 mg/m^2 on day 1, once every 3 wk, were proposed. In our study we used capecitabine 1000 mg/m^2 (bid) on days 1-14 and irinotecan 200 mg/m^2 on day 1 (with or without bevacizumab 7.5 mg/kg body weight on day 1), in accordance with Borner's study (2005) with good patient compliance[38]. Furthermore, the safety profile of the recent multicentric phase Ⅱ AIO trial with 247 patients comparing XELOX-Bev with CAPIRI-Bev with a dose of capecitabine 800 mg/m^2, which was 200 mg/m^2 less than used in our trial[39,40] was favorably comparable with the number of adverse events reached in our study. Moreover, the group reported an incidence of Grade 3-4 thromboembolic events of 5% and hypertension of 3%, where these events were not observed in our trial. Similarly, Hochster *et al*[27] reported from both TREE studies a decrease in Grade 3-4 toxicities of approximately 10%-20% for oxaliplatin-based regimens in combination with bevacizumab, which compares well with the decrease of approximately 25% reached by the addition of bevacizumab to CAPIRI in our study.

The overall incidence of grade 3-4 toxicity was higher with CAPIRI than with the CAPIRI-Bev combination: 82.4% *vs* 58.6%, respectively. The grade 3-4 diarrhea, which is the most common dose-limiting toxicity with CAPIRI, was also reduced in those patients receiving CAPIRI-Bev: 29.4% *vs* 17.3%, respectively. This is in clear concordance with recent efficacious and tolerable data on CAPIRI [capecitabine 1000 mg/m^2 (bid), days 1-14; irinotecan 250 mg/m^2 on day 1] in 398 patients, which did not to replicate the toxicity and mortality observed in earlier phase Ⅲ studies incorporating high doses of irinotecan[36,41,42].

In summary, these data are preliminary and should be treated with caution due to the small numbers of patients studied, and because the patients were not randomized to either treatment arm, neither was the study statistically set up to compare treatments. However, taken together, both regimens-CAPIRI alone or with the addition of bevacizumab-were well tolerated and are useful for efficient tumor control in out-patient settings of non-university based out-patient clinics, with the emphasis of patients' recommendations for its possible side effects. Severe gastrointestinal and thromboembolic events seen in other studies[43,44] were rarely observed and never fatal in our study. Thus, many authors concluded that combinations of CAPIRI with both, VEGF or EGFR1 antibodies seemed to be safe and feasible[39].

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COMMENTS

Background

Novel combinations of new chemotherapeutic agents with the increasing number of targeted monoclonal antibodies to tumor expressed antigens, have demonstrated an improvement in tumor response and overall survival in patients with advanced colorectal cancer (CRC) when investigated in the setting of a clinical trial.

Research frontiers

Capecitabine, an oral fluoropyrimidine, irinotecan a topoisomerase inhibitor, and the vascular endothelial growth factor (VEGF) bevacizumab, have demonstrated improved efficacy and tolerable toxicity when compared to with standard therapy following randomized trials in advanced colorectal cancer patients. However few studies have investigated their use in community-based hospital outpatient clinics where many patients with the disease are typically treated.

Innovations and breakthroughs

This is one of the first studies of its kind to investigate capecitabine in combination with irinotecan (CAPIRI) with and without bevacizumab in a community-based hospital out-patients clinic. The data suggest that both treatment regimens demonstrate efficacy and tolerable toxicity in this setting.

Applications

These data are in accordance with the preliminary findings emerging from larger community-based trials which are examining many different treatment combinations in advanced CRC. The data suggest that the improvements in efficacy and safety observed in trials performed in experienced university-based settings in selected patients will be in some cases translated to patients treated in the routine community-based setting, where experience of the use of such new agents is limited, but a substantial proportion of patients with this disease are treated.

Terminology

Capecitabine is an orally administered derivative of 5-fluorouracil (5-FU), sometimes given in preference to intravenously delivered 5-FU due to its ease of administration. It kills tumor cells by preventing DNA synthesis through the inhibition of thymidylate synthase, which is active in proliferating cells. Irinotecan also kills cells by inhibiting the enzyme topoisomerase I which is also involved in DNA synthesis in proliferating cells. Bevacizumab is a monoclonal antibody that inhibits VEGF that is expressed in some CRC aiding the blood supply to tumors; bevacizumab inhibits this process, stopping the tumor cells from growing.

Peer review

This is a non randomized study comparing the efficacy and safety of CAPIRI with and without bevacizumab in the treatment of patients attending community based clinical out-patients clinics. The preliminary results are interesting and may confirm the usefulness of CAPIRI with or without bevacizumab in this clinical setting.

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Risk factors for colonic diverticular bleeding: A Westernized community based hospital study

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Abstract

AIM: To evaluate the risk factors-other than nonsteroidal anti-inflammatory drugs-for colonic diverticular bleeding in a westernized population.

METHODS: One hundred and forty patients, treated for symptomatic diverticular disease in a community based hospital, were included. Thirty (21%) had signs of diverticular bleeding. Age, gender, and the results of colonoscopy were collected and compared to a group of patients with nonbleeding symptomatic diverticulosis. Records were reviewed for comorbidities, such as obesity, alcohol consumption, smoking habits and metabolic diseases. Special emphasis was put on arterial hypertension, cardiovascular events, diabetes mellitus, hyperuricemia and hypercholesterinemia.

RESULTS: There was no difference between patients with diverticular hemorrhage and those with nonbleeding symptomatic diverticulosis regarding gender ratio (male/female 9/21 *vs* 47/63) and diverticular localisation. Bleeding patients differed in respect to age (73.4 ± 9.9 *vs* 67. 8 ± 13.0, $P < 0.013$). Significant differences were found between both groups regarding the presence of hyperuricemia and use of steroids and nonsteroidal anti-inflammatory drugs. Patients with three concomitant metabolic diseases were also identified as being at risk of bleeding. A forward stepwise logistic regression analysis revealed steroids, hyperuricemia and the use of calcium-channel blockers as independent risk factors of bleeding.

CONCLUSION: Beside nonsteroidal anti-inflammatory steroid drug use, antihypertensive medication and concomitant arteriosclerotic diseases are risk factors for colonic diverticular hemorrhage. Our results support the hypothesis of an altered arteriosclerotic vessel as the source of bleeding.

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Key words: Diverticula; Gastrointestinal bleeding; Arteriosclerosis; Risk factors; Calcium channel blocker

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INTRODUCTION

Diverticular disease is common in Westernized countries[1]. In adults under 40, less than 10% are affected. This increases to a prevalence of up to 60% in those aged over 80. Prevalence is equal between men and women. In Western countries the disease usually affects the left side of the colon[2], whereas in Eastern countries diverticulosis is more common in the right side[3]. In 80% of affected individuals, the condition remains asymptomatic. The remaining patients develop signs of diverticular disease. These include left sided lower abdominal pain, change in bowel habits and abdominal distension. Complicated diverticular disease presents as diverticulitis with perforation, obstruction, formation of abscesses and fistulation[4]. The second severe complication is the acute diverticular bleeding, which affects 3% to 15% of individuals with diverticular disease[5,6]. The pathogenesis of diverticular bleeding includes an asymmetric intimal proliferation and segmental weakening of the associated vas rectum arising from traumatic factors within the diverticular or colonic lumen. The bleeding is thought to be the

result of an acute rupture of the altered vasa recta close to the diverticulum[7]. Arteriosclerosis and associated diseases such as the metabolic syndrome could therefore play an important role in the pathogenesis of acute diverticular bleeding. Nonsteroidal anti-inflammatory drugs (NSAID), including aspirin, have already been identified as risk factors for acute lower gastrointestinal bleeding, including acute diverticular bleeding[3,8-10]. In a recently published survey, several potential risk factors for diverticular bleeding have been analysed in Japanese patients. Apart from NSAID and anticoagulants, arterial hypertension has been shown to be an independent risk factor for colonic diverticular bleeding[3]. Risk factors for diverticular bleeding in Western populations have not been explored before. We therefore analysed patients admitted to a Western community based hospital with colonic diverticular bleeding regarding comorbidities and medications as possible risk factors.

MATERIALS AND METHODS

Two hundred and one patients, treated for symptomatic diverticular disease at the medical Department of the St. Elisabeth Hospital, Dorsten, between January 2004 and July 2006, were included in this study. Overall, 140 patients with proven diverticulosis and colonoscopy during the hospital stay were reviewed retrospectively. The remaining patients either refused endoscopy or went directly to surgical intervention. None of the latter patients suffered from intestinal bleeding.

Thirty of the 140 investigated patients had signs of lower intestinal bleeding on admission. Data from these patients was compared to data from patients without signs of lower gastrointestinal bleeding.

Data collection

We collected data on age, gender and results of colonoscopy. The records were also reviewed for information on comorbidities, ongoing medication, body mass index (BMI), alcohol consumption and smoking habits. Special emphasis was put on concomitant diseases such as arterial hypertension, cardiovascular events (stroke, coronary heart disease), diabetes mellitus including prediabetes (impaired glucose utilization), hyperuricemia and hypercholesterolemia. In addition, outcome, development of complications and length of hospital stay were compared. Concomitant diseases were assumed when patients were on appropriate medication. Obesity was defined as BMI > 25 kg/m^2.

Colonoscopy

Colonoscopy was performed after using polyethylene glycol-containing lavage solution for colon preparation by use of electronic video endoscopes (Model Exera CF-Q 145/160, Olympus Optical Co, Hamburg, Germany). Diagnosis of colonic diverticular hemorrhage was made based on the criteria reported by Jensen *et al*[6]. These criteria include the observation of active bleeding from a diverticulum, the observation of blood clots in the colon in the presence of diverticula, absence of blood in the terminal ileum, and no other demonstrable cause of bleeding.

Table 1 Characteristics of patients with and without colonic diverticular hemorrhage *n* (%)

	Diverticular hemorrhage	Nonbleeding diverticular	*P* value
No	30	110	
Age (yr, mean ± SD, range)	73.4 ± 9.9 (50-90)	67.8 ± 13.0 (40-93)	0.013
Male/Female ratio	9/21	47/63	NS
Diverticular location			
Left side	22 (73.3)	84 (77.8)	NS
Bilateral	8 (26.7)	24 (22.2)	

Data are mean ± SD; NS: Non significant.

Statistical analysis

The data were evaluated using descriptive statistical methods (mean ± SD, ranges). For comparison of two means the unpaired Students's *t*-test was used. Frequency distribution was calculated by the χ^2 test or Fisher's exact probability test. Factors with $P < 0.2$ in the univariate analysis were included in a forward stepwise logistic mulitivariante regression analysis. Statistical calculations were performed using SPSS Version 16.0 for Windows (SPSS Inc Chicago, USA).

RESULTS

The characteristics of patients with and without colonic diverticular hemorrhage are shown in Table 1. While gender distribution was comparable, patients with diverticular bleeding were significantly older. None of our patients had isolated right-sided colonic diverticula. Isolated left-sided colonic affection was most frequent in both groups.

Colonoscopy was performed in the bleeding group after 3.8 ± 2.3 d (range 1-9) and in the nonbleeding group after 6.0 ± 3.7 d (range 1-28, $P = 0.003$). Colonoscopy was successful in 83% of bleeding patients and in 79% of nonbleeding patients. The reasons for early termination were obstruction in 10% of patients in the bleeding group and 17% in the nonbleeding group. In the remaining patients colonoscopy was incomplete because of inappropriate cleaning or pain.

Endoscopic signs of diverticular inflammation (petechial bleeding, pus, reddening, and swelling) were found in 63.3% of patients in the bleeding group and 59.1% of patients in the nonbleeding group (NS).

Since polyps are also potential sources of bleeding we compared the prevalence of such lesions in both groups. In the bleeding group polyps were found in seven patients (23%) whereas, in the nonbleeding group, 29 patients (26%, NS) had polyps. None of the polyps showed signs of recent hemorrhage.

The definitive source of bleeding was identified in nine (30%) patients (bleeding vessels, nonbleeding

Table 2 Potential risk factors for diverticular bleeding *n* (%)

	Diverticular hemorrhage	Nonbleeding diverticular	*P* value
No	30	110	
Comorbidities			
BMI (kg/m^2, mean ± SD, range)	28.2 ± 5.0 (21-43)	26.7 ± 5.0 (13-46)	0.15
Obesity (BMI > 25 kg/m^2)	20 (69.0)	61 (59.2)	0.34
Diabetes mellitus	7 (23.3)	15 (13.6)	0.2
Hypertension	20 (66.7)	65 (59)	0.45
Hyperlipoproteinemia	8 (26.7)	25 (22.7)	0.65
Hyperuricemia	6 (20)	8 (7.3)	0.039
Smoking	2 (6.7)	17 (15.5)	0.21
Alcohol	0	5 (4.6)	0.23
Immunodeficiency[1]	4 (13.3)	9 (8.2)	0.39
Cardiovascular events[2]	10 (33.3)	28 (25.5)	0.39
Medication			
Steroids	4 (13.3)	3 (2.7)	0.018
NSAR	4 (13.3)	4 (3.6)	0.043
Cumarine	2 (6.7)	4 (3.6)	0.47
Aspirin	9 (30.0)	30 (27.3)	0.77
β-blockers	7 (23.3)	33 (30.0)	0.47
ACE-inhibitors	6 (20.0)	25 (22.7)	0.75
AT-Ⅱ-receptor-antagonists	0	10 (9.1)	0.087
Calcium antagonist	10 (33.3)	23 (20.9)	0.15
Digitalis	5 (16.7)	7 (6.4)	0.074

[1]Including malignant disease, chemotherapy, regular intake of methotrexate or steroids, reduced general condition due to old age. [2]Including stroke and myocardial infarction.

vessel in the diverticulum and adherent clot in the diverticulum). The bleeding was localized in the right colon in one patient and in the left colon in the remaining patients. Colonoscopy was performed with these patients within three days (1.8 ± 0.7 d), whereas patients without definitive signs of bleeding were examined between day two and nine after admission (4.7 ± 2.2, $P < 0.001$).

To identify potential risk factors for diverticular bleeding we compared bleeding and nonbleeding patients regarding comorbidities and medications. The results are shown in Table 2. Hyperuricemia was present significantly more often in patients with diverticular bleeding. All other comorbities showed no significant differences. However, patients were more likely to develop diverticular bleeding when more than three concomitant diseases, out of seven (obesity, hypertension, hypercholesterolemia, hyperuricemia, impaired glucose utilization, arteriosclerosis, immunosuppression), were present ($P = 0.013$). By univariate analysis, steroid and NSAID use were also increased in patients with bleeding. In addition, diabetes mellitus, BMI, calcium channel blockers and digitalis had a P value < 0.2. Using these factors, we performed a multivariate forward logistic regression analysis: steroid use ($P = 0.01$), hyperuricemia ($P = 0.004$), and use of calcium channel blockers ($P = 0.03$) were confirmed independent factors of bleeding.

There was no difference in length of hospital stay between the bleeding and the nonbleeding group. During hospital stay, four patients from the bleeding group (13.3%) and eight from the nonbleeding group (7.3%, P = NS) required surgery.

Severity of diverticular hemorrhage was classified according to the initial RBC in 28 patients. Patients with an initial hemoglobin level lower than 10 g/dL were considered to have more severe bleeding. These patients were older than those with a less severe bleeding (81 ± 4 *vs* 72 ± 10 years, $P = 0.037$). All other potential risk factors showed no differential distribution between the groups, although trends could be seen in steroid use, NSAID use and cardiovascular events.

Length of hospital stay differed between severe and non-severe bleeding patients (17 ± 10 d *vs* 10 ± 7 d, $P = 0.067$). Two patients in the severe group died due to cardiovascular events and multiple organ failure. No patient of the non-severe group died.

DISCUSSION

Our study shows that the risk of diverticular bleeding increases with age. In particular, higher age is associated with a more severe bleeding. Steroid and NSAID use predisposes to diverticular bleeding. Hyperuricemia, steroid and calcium channel blockers use are independent risk factors for bleeding out of a variety of ateriosclerotic diseases and medications. A higher bleeding risk is also associated with the concomitant presence of more than three of such conditions.

The prevalence of diverticular disease, in general, increases with age[5]. Complicated diverticulitis, including obstruction, perforation and abscess formation leading to operation, however, is more likely in patients younger than 50 years of age with coexisting conditions, including obesity[11]. Our results show that in contrast diverticular bleeding is an event which occurs in older age. In addition, obesity has no influence. The association of obesity and diverticular disease is controversial. A slight association between elevated BMI and symptomatic diverticular disease was reported by Aldoori *et al*[12], whereas other authors did not find such a correlation[13,14]. In our study the prevalence of obesity in the bleeding and nonbleeding group was comparable to the reported prevalence of obesity in the general population of this urban area[15]. Thus, the presence of diverticula and the occurrence of a bleeding episode are both independent of obesity.

Diverticular bleeding and complicated diverticular disease are of different pathogenesis. This is supported by our observation that patients with bleeding had no higher frequency of inflammation than patients without bleeding[16]. Diverticular bleeding is thought to be the result of a rupture of an arteriosclerotic altered diverticular vessel[7,17]. Since arteriosclerosis increases with age, age itself might not be an independent risk factor for bleeding[13]. Instead, arteriosclerotic disease could be the true risk factor for bleeding. In our study,, we could not find an association of bleeding with a single arteriosclerotic disease. Arteriosclerosis is also the result of different metabolic diseases. Therefore we speculate that the presence of these diseases increases

the risk of bleeding. Our results show that this is only true for hyperuricemia, but not for other metabolic diseases, including obesity. A recent survey on Japanese patients confirms our results, that arteriosclerosis in general and obesity are not single independent risk factors for bleeding. However, these authors identified arterial hypertension as the only independent risk factor for hemorrhage[3]. Although in our study arterial hypertension is not associated with a higher bleeding risk, hyperuricemia is. Yamada *et al*[3] speculate from their findings that microscopic hemorrhages occur frequently in diverticulosis and progress into severe bleeding in the presence of unfavorable conditions, such as arterial hypertension. This speculation is supported by the finding that NSAID use, by causing microscopic bleeding, also increases the risk of significant diverticular bleeding.

From our results we can conclude that, beside arterial hypertension and NSAID use, additional conditions, like hyperuricemia and steroid use, are additional risk factors of bleeding. Furthermore, the combination of several arteriosclerotic risk factors is also associated with a higher bleeding risk. This supports the hypothesis that vascular fragility is increased in elderly patients, and especially in patients with multiple concomitant diseases. NSAIDs reduce mucosal prostaglandin production, which leads to enhanced mucosal permeability and reduced microcirculation, and, in turns, to mucosal inflammation and bleeding[18,19].

In our study, hyperuricemia was a risk factor for diverticular bleeding. This fits into the concept that metabolic diseases leading to arteriosclerosis could cause the bleeding episode. And indeed, uric acid has been identified as a risk factor for stroke and myocardial infarction[20]. In our study, hyperuricemia was assumed when patients were on adequate medication (allopurinol). Thus we cannot exclude that allopurinol itself could have an influence on diverticular bleeding. Calcium channel blockers use was also associated with a higher bleeding risk. An elevated risk for gastrointestinal bleeding is known to be present in older adults following calcium channel blocker use[21,22]. Thus, our observation shows that diverticula are a possible source of bleeding in these patients. Therefore, it should be kept in mind that older patients with arterial hypertension and diverticula have an elevated risk for gastrointestinal bleeding when treated with calcium channel blockers.

In our study, most patients suffered from left sided diverticular disease. Accordingly, this was also the most frequent localisation of the bleeding diverticulum. This is in contrast to the general opinion where right sided diverticula are thought to be the most frequent source of bleeding. However there are only limited data which support this hypothesis. Even a recent Japanese study could not find such an association[3].

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COMMENTS

Background

Diverticular disease of the colon is common in Westernized countries. One of its complications is bleeding. Our study tried to identify the risk factors for this event.

Research frontiers

To know potential risk factors for bleeding is essential for treating patients with diverticulosis and concomitant diseases.

Innovations and breakthroughs

In Westernized countries, the risk factors for diverticular bleeding are the use of nonsteroidal anti-inflammatory drugs (NSAID), the use of steroids, antihypertensive medications and concomitant arteriosclerotic diseases. Anticoagulation and antiplatelet therapies were not identified as risk factors.

Applications

Patients with known diverticula should avoid NSAID, and during antihypertensive medication the risk for diverticula bleeding should be kept in mind.

Terminology

Diverticula are herniations of the muscosa and submucosa through the muscular coat of the colon. There is a close relationship between the herniated sac and the arterial supply. This is of importance for the pathophysiology of bleeding.

Peer review

It is a well written paper, substantiating evidence for some known risk factors and identifying some new, like hyperuricemia, and use of calcium channel blocker.

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BRIEF ARTICLES

Clinical expression of insulin resistance in hepatitis C and B virus-related chronic hepatitis: Differences and similarities

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Abstract

AIM: To investigate the prevalence of the clinical parameters of insulin resistance and diabetes in patients affected by chronic hepatitis C (CHC) or chronic hepatitis B (CHB).

METHODS: We retrospectively evaluated 852 consecutive patients (726 CHC and 126 CHB) who had undergone liver biopsy. We recorded age, sex, ALT, type 2 diabetes and/or metabolic syndrome (MS), body mass index (BMI), and apparent disease duration (ADD).

RESULTS: Age, ADD, BMI, prevalence of MS and diabetes in patients with mild/moderate liver fibrosis were significantly higher in CHC. However, the degree of steatosis and liver fibrosis evaluated in liver biopsies did not differ between CHC and CHB patients. At multivariate analysis, age, sex, BMI, ALT and diabetes were independent risk factors for liver fibrosis in CHC, whereas only age was related to liver fibrosis in CHB. We also evaluated the association between significant steatosis (> 30%) and age, sex, BMI, diabetes, MS and liver fibrosis. Diabetes, BMI and liver fibrosis were associated with steatosis > 30% in CHC, whereas only age and BMI were related to steatosis in CHB.

CONCLUSION: These data may indicate that hepatitis C virus infection is a risk factor for insulin resistance.

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Key words: Insulin resistance; Diabetes; Metabolic syndrome; Hepatitis C virus; Hepatitis B virus

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INTRODUCTION

Recent years have seen numerous studies devoted to the relationship of such clinical variables as age, gender and body mass index (BMI) with metabolic alterations involving the liver in patients affected by hepatitis C virus (HCV)-related chronic hepatitis. Attention has focused on the metabolic syndrome (MS), diabetes[1-5], steatosis/steatohepatitis[6-10] and associations between these conditions[11-15]. It remains to be established whether the relationship between HCV and deranged cellular metabolic pathways is casual or whether it results from a direct effect exerted by HCV.

In an attempt to shed light on this issue, we examined the prevalence of these phenomena in chronic hepatitis C (CHC) and in chronic hepatitis B (CHB) in order to distinguish between alterations due to presence of an advanced liver disease and those due to HCV[16-18].

We have investigated the prevalence of MS and/or diabetes and their effect on liver fibrosis and/or steatosis/steatohepatitis in a large cohort of patients affected by HCV- or hepatitis B virus (HBV)-related chronic hepatitis. Our aim was to look for differences and simi-

larities between the two types of viral hepatitis and to confirm or refute the hypothesis of metabolic cofactors in HCV-related liver disease(s).

MATERIALS AND METHODS

We retrospectively evaluated 852 consecutive patients (726 CHC and 126 CHB) who underwent clinical evaluation and liver biopsy for HBV- or HCV-related hypertransaminasemia in the Internal Medicine and Hepatology Unit at the Second University of Naples from April 1999 to November 2005. The inclusion criterion was HBsAg-positive or HCV-Ab-positive chronic hepatitis. The exclusion criteria were: patients with a recent (< 6 mo) history of high alcohol consumption (> 30 g/d in females, > 40 g/d in males) or a history of intravenous drug abuse, clinical and/or ultrasound evidence of cirrhosis, an human immunodeficiency virus (HIV)-positive test and HBV/HCV co-infection. In addition to a complete blood count, each patient underwent tests for: serum markers for hepatitis B infection determined by radioimmunoassay (Abbott Laboratories, North Chicago, IL, USA), antibodies to HCV assayed by a second-generation enzyme-linked immunoassay (ELISA; Ortho Diagnostic Systems, Raritan, NJ, USA), the presence of type 2 diabetes according to American Diabetes Association criteria[19] by assessing fasting serum glucose and/or the oral glucose tolerance test (OGTT) on two different occasions, and for the presence of MS based on NCEP-ATP Ⅲ criteria[20]. BMI (kg/m^2) was recorded and the apparent disease duration [(ADD) in years] was determined by considering the exposure to major risk factors as the start of infection. Each patient underwent an echo-assisted liver biopsy performed with a 17G Menghini modified needle (Surecut, TSK Laboratory, Japan) through an intercostal entry. The liver specimens were formalin-fixed, embedded in paraffin and evaluated with the Ishak scoring system for viral liver disease[21] by skilled liver pathologists, who also evaluated the presence of liver steatosis and steato-hepatitis according to Brunt *et al*[22].

Statistical analysis of data was performed using the SPSS® 13.0 software for Windows®. Non-parametric tests (χ^2 or Fisher's exact), Spearman's rho test, ANOVA, linear and logistic regression tests were performed where appropriate.

RESULTS

As shown in Table 1, HCV-positive patients had a significantly longer ADD ($P < 0.005$), a significantly older age ($P < 0.0001$) and a significantly higher BMI ($P < 0.005$) *vs* HBV-positive subjects. The MS was present in 76 out of 726 HCV patients (10.46%) and in only 2 of the 126 HBV subjects ($P < 0.005$). The prevalence of diabetes did not differ significantly between the two groups, but it was significantly higher in HCV subjects when comparing only patients with mild fibrosis (Ishak S0-S3) at liver biopsy ($P < 0.005$) (Figure 1). The prevalence of diabetes in mild fibrosis patients did not differ between the two groups when patients were stratified by age. No differences were found at liver biopsy as regards grading and staging, neither was there a difference in the prevalence of patients with higher degrees of steatosis.

Table 1 Epidemiological and histological data of the two groups studied

	HCV	HBV	*P*
Epidemiological data			
Number of patients (M/F)	726 (426/300)	126 (83/43)	NS
DD (yr)	25.78 ± 14.22	21.4 ± 11.91	< 0.005
Age (yr)	53.88 ± 13.20	45.68 ± 14.40	< 0.0001
BMI (kg/m^2)	26.74 ± 4.25	25.43 ± 3.43	< 0.005
Metabolic syndrome	76 pt (10.46%)	2 pt (1.72%)	< 0.005
Liver Biopsy (Ishak Score)			
Grading	11.25 ± 3.50	6.61 ± 3.36	NS
Staging	2.66 ± 1.99	2.44 ± 1.57	NS
Steatosis > 30%	98 pt (13.49%)	14 pt (12.06%)	NS

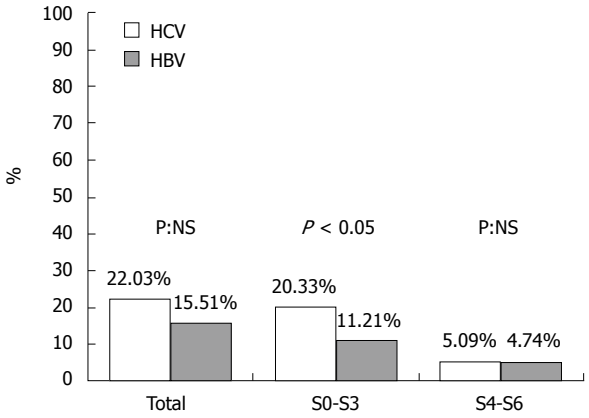

Figure 1 Diabetes prevalence divided by fibrosis score (Ishak).

To evaluate whether age, sex, BMI, ALT, diabetes, MS and steatosis were independent risk factors for liver fibrosis we carried out a univariate and a multivariate analysis in the two groups of patients. At univariate analysis, all the variables tested were significantly related to higher degrees of fibrosis in HCV patients, whereas only age was correlated to liver fibrosis in HBV patients (Table 2). The results of the multivariate analysis confirmed these findings, namely age, sex, BMI, ALT and diabetes were independent risk factors for fibrosis in HCV subjects, whereas only age was related to fibrosis in HBV patients (Table 3).

We next carried out statistical analyses to evaluate whether age, sex, BMI, ALT or diabetes were related to steatosis, which is a well known negative predictor of both disease outcome and response to therapy in HCV patients[23-26]. None of these parameters were significantly associated with degree of steatosis. However, when we did the same analysis considering only patients with steatosis > 30%, a higher BMI, diabetes and fibrosis were correlated with severe steatosis in CHC patients (Tables 4 and 5).

DISCUSSION

In our cohort study, the prevalence of MS and diabetes was significantly higher in patients with HCV-related chronic hepatitis than in patients with HBV-related

Table 2 Univariate analysis (Spearman test) between liver fibrosis (Ishak staging on liver biopsy) and anthropometric, clinical and metabolic characteristics in the two groups of patients

	HCV				HBV			
	Linearity coefficient	Standard error	*P*	*t*	Linearity coefficient	Standard error	*P*	*t*
Age (yr)	0.332	0.046	< 0.0001	7.377	0.532	0.071	< 0.0001	5.689
Sex	0.284	0.046	< 0.05	3.764	0.132	0.108	NS	1.209
BMI	0.243	0.050	< 0.0001	4.633	0.142	0.113	NS	1.162
ALT	0.227	0.045	< 0.0001	4.833	0.157	0.082	NS	1.435
Diabetes	0.211	0.050	< 0.0001	4.532	0.014	0.123	NS	0.103
Metabolic syndrome	0.108	0.039	< 0.05	2.278	0.015	0.011	NS	0.112
Steatosis	0.095	0.045	< 0.05	2.006	0.068	0.187	NS	0.283

Table 3 Multivariate analysis (multiple regression) of the correlation between liver fibrosis (Ishak staging on liver biopsy) and anthropometric, clinical and metabolic characteristics in the two groups of patients

	HCV			HBV		
	Linearity coefficient	Standard error	*P*	Linearity coefficient	Standard error	*P*
Age (yr)	0.0469	0.0068	< 0.0001	0.0561	6.0142	0.0001
Sex	0.0362	0.1794	< 0.05	-	-	-
BMI	0.0417	0.0208	< 0.05	-	-	-
ALT	0.0038	0.0010	< 0.0001	-	-	-
Diabetes	0.4742	0.2172	< 0.05	-	-	-
Metabolic syndrome	0.2830	0.2846	NS	-	-	-
Steatosis	0.0731	0.2326	NS	-	-	-

Table 4 Univariate analysis of the correlation between the presence of higher degrees of steatosis (> 30%) assessed on liver biopsy and anthropometric, clinical and metabolic characteristics in the two groups of patients

	HCV			HBV		
	OR	95% CI	*P*	OR	95% CI	*P*
Age (yr)	1.747	0.888-3.430	NS	4.432	1.056-18.310	< 0.05
Sex	0.845	0.551-1.294	NS	0.549	0.191-1.575	NS
BMI (> 30)	2.510	1.593-3.954	< 0.001	10.435	2.166-38.338	< 0.005
Diabetes	1.894	1.193-3.007	< 0.05	0.913	0.213-4.017	NS
Metabolic syndrome	0.968	0.486-1.931	NS	4.180	0.531-3.367	NS
Fibrosis (staging)	4.700	2.750-8.034	< 0.001	0.459	0.073-2.981	NS

Table 5 Multivariate analysis (logistic regression) of the correlation between the presence of significant steatosis (> 30%) assessed on liver biopsy and anthropometric, clinical and metabolic characteristics in the two groups of patients

	HCV			HBV		
	OR	95% CI	*P*	OR	95% CI	*P*
Age (yr)	-	-	-	1.214	1.085-1.359	< 0.001
BMI (> 30)	2.216	1.186-4.143	< 0.05	1.049	1.007-1.094	< 0.05
Diabetes	2.684	1.444-4.987	< 0.005	-	-	-
Fibrosis (Staging)	4.700	2.750-8.034	< 0.001	-	-	-

chronic liver disease. This suggests that insulin resistance, which is the mechanism underlying MS, might at least in part be related to HCV infection. Insulin resistance is identified with the euglycemic clamp technique[27] or with the homeostasis model assessment insulin resistance (HOMA-IR) test[28]. Neither euglycemic clamp nor the HOMA-IR test was performed in our patients. However, we used the most practical worldwide accepted definition of MS according to the ATP-Ⅲ NCEP clinical criteria[20]. Moreover, unlike other authors[16,29-32], we were able to measure, using these criteria, central obesity which is considered the fundamental condition for a clinical diagnosis of MS[33]. The association of diabetes and severe chronic liver disease is widely recognized[4,16,32,34], therefore the effect of severe liver disease on diabetes onset should be considered when comparing groups of patients affected by chronic liver disease. In our study, we were able to rule out that diabetes was related to the altered hepatic glycaemic homeostasis due to severe liver disease (i.e. cirrhosis). In fact, whereas the total prevalence of diabetes did not differ between HCV and HBV patients, the prevalence of diabetes among patients with mild chronic hepatitis was significantly higher in HCV subjects.

This finding confirms reports of a higher prevalence of diabetes in HCV patients[14,29,34], and, *vice versa*, a report of a higher prevalence of HCV in diabetic patients[30]. Moreover, our data are in line with recent reports of a higher prevalence of diabetes in HCV patients compared with other liver diseases (e.g. HBV infection)[1]. In particular, Jan *et al*[17] reported a significantly higher prevalence of MS in HCV patients *vs* HBV patients, and an inverse ratio between HBV infection and clinical expression of MS in a population-based study carried out in Taiwan.

The association between diabetes and HCV-related chronic hepatitis was reported to influence the clinical outcome of liver disease[35-37]. Moreover, diabetes and/or obesity were found to be cofactors of liver disease in that they affect liver fibrosis progress and response to antiviral therapy[23,24,37-39]. Here we report that, besides ALT, sex and age, also diabetes and BMI were independent risk factors for more severe liver fibrosis in HCV-positive patients, whereas they were not associated with the severity of liver fibrosis in HBV subjects. This finding supports the idea that obesity and diabetes might represent clinical epiphenomena of the pathogenesis of HCV infection. In line with this hypothesis, using an animal model, Shintani *et al*[40] found that HCV directly affects serum glycaemic

and insulinaemic levels, probably by acting *via* TNF-α. Similarly, Kawaguchi *et al*[41] reported that HCV directly affects intracellular insulin metabolic pathways *via* genetic up-regulation. However, we cannot exclude the possibility that synergism between the two diseases (diabetes/non-alcoholic steatohepatitis and virus-related chronic hepatitis) affects the outcome of liver fibrosis[7,11].

Steatosis has been associated with more severe liver fibrosis[23-26] as well as with a worse response to antiviral therapy. Moreover, steatosis is defined "metabolic steatosis" in non-3-genotype-infected patients as opposed to the viral steatosis typical of genotype 3 patients[11]. In our cohort of HCV patients, steatosis did not appear to be an independent risk factor for fibrosis stage. The discrepancy between our results and previous findings is apparent. Indeed, univariate and multivariate analyses *vs* fibrosis showed that steatosis was associated with the severity of liver fibrosis in our HCV patients if they were also affected by diabetes and/or obesity. Moreover, at multivariate analysis *vs* steatosis, BMI, diabetes and fibrosis were independent risk factors only for steatosis > 30%. This suggests that histological features of fibrosis, whether related to non-alcoholic steatohepatitis or to virus-associated steatohepatitis, will be found only in cases in which steatosis is > 30%.

This study provides clinical evidence that HCV directly affects insulin resistance in a large Italian retrospective, single-centre, consecutive population of HCV- and HBV-infected subjects. Biological, molecular and genetic studies are required to test this hypothesis.

COMMENTS

Background

Glucose metabolism derangements are common in hepatitis C virus (HCV) patients but there is still a lot of deliberation as to whether the relationship between HCV and deranged cellular metabolic pathways is casual or whether it results from a direct effect exerted by HCV.

Research frontiers

To investigate the prevalence of the clinical parameters of insulin resistance and diabetes in patients affected by chronic hepatitis C (CHC) or chronic hepatitis B (CHB).

Innovations and breakthroughs

A direct comparison of the prevalence of clinical features of insulin resistance in HCV and HBV infection in a large cohort of Italian patients.

Applications

To shed light on the association between insulin resistance and HCV infection and recommend further biological, molecular and genetic studies, and finally to suggest a careful assessment of insulin resistance status in HCV clinical care.

Peer review

The authors retrospectively analyzed a large cohort of consecutively-enrolled HCV (726) and HBV (126) patients undergoing liver biopsy in order to find a correlation between clinical expressions of insulin resistance (diabetes and metabolic syndrome) and HCV liver disease in comparison with HBV-related chronic hepatitis. This is an interesting paper and is of importance.

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Effect of preoperative immunonutrition and other nutrition models on cellular immune parameters

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Abstract

AIM: To evaluate the effects of preoperative immunonutrition and other nutrition models on the cellular immunity parameters of patients with gastrointestinal tumors before surgical intervention. In addition, effects on postoperative complications were examined.

METHODS: Patients with gastrointestinal tumors were randomized into 3 groups. The immunonutrition group received a combination of arginine, fatty acids and nucleotides. The second and third group received normal nutrition and standard enteral nutrition, respectively. Nutrition protocols were administered for 7 d prior to the operation. Nutritional parameters, in particular prealbumin levels and lymphocyte subpopulations (CD4+, CD8+, CD16+/56+, and CD69 cells) were evaluated before and after the nutrition protocols. Groups were compared in terms of postoperative complications and duration of hospital stay.

RESULTS: Of the 42 patients who completed the study, 16 received immunonutrition, 13 received normal nutrition and 13 received standard enteral nutrition. prealbumin values were low in every group, but this parameter was improved after the nutritional protocol only in the immunonutrition group (13.64 ± 8.83 *vs* 15.98 ± 8.66, P = 0.037). Groups were similar in terms of CD4+, CD16+/56, and CD69+ prior to the nutritional protocol; whereas CD8+ was higher in the standard nutrition group compared to the immunonutrition group. After nutritional protocols, none of the groups had an increase in their lymphocyte subpopulations. Also, groups did not differ in terms of postoperative complications and postoperative durations of hospital stay.

CONCLUSION: Preoperative immunonutrition provided a significant increase in prealbumin levels, while it did not significantly alter T lymphocyte subpopulation counts, the rate of postoperative complications and the duration of hospital stay.

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Key words: Malnutrition; Gastrointestinal tumours; Immunonutrition; Prealbumin; Lymphocyte subpopulations

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INTRODUCTION

Protein-energy malnutrition occurs in 30% to 90% of patients with cancer[1]. Malnutrition causes adverse effects on immunity through several mechanisms, including atrophy in lymph nodes, decreased lymphocyte count and IgA production and suppression of cellular immunity. Many studies have shown beneficial effects of nutritional support in patients with malnutrition[2-4].

Immunonutrient compounds with a suggested positive effect on immune parameters such as arginine, glutamine, omega 3 fatty acids, and ribonucleic acid are now being studied with regard to their contribution to immune response when given as supplements to nutritional treatments[5-8].

Immunonutrition is usually given preoperatively, since it prevents the decrease in cellular immunity and phagocytic capacity of polymorphonuclear neutrophils (PMN's) during the early postoperative period[9]. There have been several prospective studies examining the effects of immunonutrition support on the rate of postoperative complications, infection rates, length of hospital stay, wound repair, weight gain, cost, and mortality[10-15]. However, the mechanisms through which beneficial effects occur have not been studied in detail. The basic immune response in the host against the tumor is mediated by cellular immunity. Studies looking at the changes in cellular immunity after immunonutrition-especially those that occur before surgical trauma- are scarce[9,16,17].

In this prospective randomized study, we examined different nutritional models in preoperative patients with endoscopically and histopathologically documented gastrointestinal tumors. The effects on nutritional parameters, quantitative measures including changes in lymphocyte subpopulations responsible for the cellular immunity [CD4+ (T helper), CD8+ (T cytotoxic, suppressor), CD16+/56+ (natural killer) cells], and qualitative measures such as changes in the expression of CD69+ were explored in immunonutritional and other nutritional models. Our objective was to avoid the possible effects of the inflammatory process due to surgical trauma on the results by evaluating the preoperative effect of immunonutrition on immunological parameters.

MATERIALS AND METHODS

A total of 56 patients with gastrointestinal tumors admitted to the General Surgery Unit of Haydarpasa Numune Hospital were included in this study. Patients with diabetes mellitus, renal and/or hepatic failure, and active infection were excluded, as were the patients with a history of immunosuppressive drug use or clinical signs of vitamin or trace element deficiency.

Informed consent was obtained from all patients. Height, weight, and midarm muscle circumference measurements were made, and weight changes in the previous six months period were evaluated. PPD and cytometric measurements were used to evaluate the cellular response. C-Reactive Protein (CRP), an acute phase reactant, and prealbumin levels were measured to demonstrate and monitor any effect that may be due to the inflammatory process.

Subjective global assessment (SGA) was based on history taking and physical examination, and patients were categorized as follows: normal nutritional status, or mild, moderate, or severe malnutrition.

Body mass index was calculated as follows: BMI = kg/m^2, weight in kilograms and height in meters. The midarm muscle circumference, forearm circumference (C) and triceps skin fold thickness (TST) were measured and calculated according to the modified Heymsfield formula[18].

Table 1 Distribution of patients by nutrition models

	Patients (*n*)	Nutrition model	Nutritional products[1]
Group 1	*n* = 16	Immunonutrition	Enteral product with addition of arginine, omega-3 fatty acids and RNA (IMPACT®)
Group 2	*n* = 13	Normal nutrition	Normal feeding planned by a dietitian
Group 3	*n* = 13	Standard enteral nutrition	Standard enteral product without RNA or Omega-3 fatty acids (FRESUBIN®)

[1]One liter of Impact (Novartis Nutrition, Bern, Switzerland) contains 780 non-protein calories, 43 g of protein, 12.5 g of arginine, 3.3 g of ω-3 fatty acids and 1.2 g of RNA.

Before nutrition, blood samples were obtained for the measurement of hemoglobin, albumin, prealbumin, CRP, and lymphocyte, CD4+, CD8+, CD16+/56+, CD69+ counts.

T-lymphocyte subpopulations were determined with immunofluorescent stained mouse anti-human monoclonal antibodies. At least 5000 cells from each subpopulation were evaluated in the flow cytometry device (Becton Dickinson, Mountainview, Ca, USA) using the Cell Quest program. For this purpose, lymphocytes were initially plotted according to their size and granularity. The accuracy of lymphocyte frames was confirmed by using CD45 FITC and CD14 FE monoclonal antibodies. CD45(+) and CD14(-) cells formed the basis for the assessments. All other cell surface markers were evaluated within this context. In another tube, cells were labeled with CD69 for the qualitative assessment of T lymphocyte responsiveness. Some of these cells were counted in flowcytometry, and others were incubated with phytohemaglutinin for 2.5 h. Then cells were re-counted in flowcytometry and the changes in CD69 expression before and after the stimulation were recorded.

Subsequently, patients were randomized to one of the preoperative nutrition models shown in Table 1 for 7 d. The energy needs were calculated using Harris-Benedict formula. For patients not receiving standard enteral nutrition, an isonitrogenous and isocaloric feeding was provided. The ratio of non-protein energy to nitrogen was 1/142 in the standard enteral product and 1/78 in the immunonutrition group.

The efficacy of nutritional support was evaluated using CRP and prealbumin measurements on days 4 and 7, and nitrogen balance on day 4. On the morning of day 8, blood samples were obtained for the assessment of the changes in nutritional parameters before patients were operated. In patients undergoing radical surgical procedures, duration of the procedure, blood loss, infection rate and duration of hospitalization

Table 2 Patient characteristics and distribution (mean ± SD)

	IM	NN	SE	*P*
Number of patients	16	13	13	
F/M ratio	(8/8)	(9/4)	(8/5)	
Age	64.56 ± 16.16	64.38 ± 11.67	61.31 ± 12.13	> 0.05
BMI	24.11 ± 3.67	23.02 ± 5.66	22.24 ± 4.87	> 0.05
Malnutrition index	7.96 ± 1.36	7.25 ± 0.79	8.40 ± 1.68	> 0.05
Muscle circumference	22.34 ± 3.01	21.11 ± 4	20.41 ± 2.31	> 0.05
Strength of hand grasping	0.46 ± 0.20	0.40 ± 0.15	0.41 ± 0.18	> 0.05
Albumin	3.6 ± 0.59	3.46 ± 0.57	3.26 ± 0.51	> 0.05
Prealbumin	13.64 ± 8.83	15.71 ± 6.97	17.72 ± 8.39	> 0.05
Number of lymphocytes	1454 ± 462	1281 ± 779.2	1277 ± 546.4	> 0.05
Subjective global assessment (SGA)[1]				
Moderate malnutrition	9	7	6	
Severe malnutrition	7	6	7	

[1]No statistical evaluation was made for subjective global assessment; IM: Immunonutrition; NN: Normal nutrition; SE: Standard enteral nutrition.

Table 3 Changes in prealbumin values with repeated measurements (mean ± SD)

Prealbumin[1]	IM	NN	SE	*P*
Baseline	13.64 ± 8.83	17.72 ± 8.39	15.71 ± 6.97	0.414
Day 4	17.12 ± 9.13	18.34 ± 6.71	16.24 ± 7.20	0.792
Day 8	15.98 ± 8.66	18.13 ± 6.76	16.41 ± 7.81	0.750
P	0.037	0.834	0.876	

[1]Normal value of prealbumin: 20-40 mg/dL; IM:Immunonutrition; NN: Normal nutrition; SE: Standard enteral nutrition.

Table 4 Percentage of lymphocyte subgroups in the peripheral blood of patients before nutritio

	IM	NN	SE	*P*
CD4 (*n* = 32%-54%)	46.13 ± 10.69	41.46 ± 9.11	49.92 ± 7.30	< 0.05
CD8 (*n* = 34%-37%)	36.81 ± 7.08	46 ± 11.34	42.08 ± 10.4	< 0.05
CD4/8 (*n* = 0.8-1.8)	1.32 ± 0.44	1.01 ± 0.54	1.26 ± 0.38	< 0.05
CD69	4.25 ± 5.6	1.40 ± 1.09	2.02 ± 2.01	> 0.05
CD69F	12.95 ± 13.05	2.70 ± 1.73	17.31 ± 10.69	< 0.001
CD16/56 (*n* = 8%-22%)	4.45 ± 3.74	7.30 ± 5.54	5.81 ± 6.38	> 0.05
CD4+, CD8+ (*n* = ≤ 1%)	4.26 ± 2.03	3.61 ± 1.80	4.08 ± 2.43	> 0.05

IM: Immunonutrition; NN: Normal nutrition; SE: Standard enteral nutrition.

Table 5 Percentage of lymphocyte subgroups in the peripheral blood of patients after nutrition (mean ± SD)

	IM	NN	SE	*P*
CD4	44.5 ± 10.09	41.62 ± 10.97	48.08 ± 12.98	> 0.05
CD8	41.5 ± 10.02	46.08 ± 12.77	40.23 ± 14.95	> 0.05
CD4/8	1.33 ± 0.56	1.04 ± 0.63	1.54 ± 1.34	> 0.05
CD69	9.22 ± 24.36	1.73 ± 1.06	3.02 ± 4.41	> 0.05
CD69F	23.11 ± 27.74	3.23 ± 1.68	14.3 ± 13.16	< 0.001
CD16/56	5.63 ± 5.05	7.52 ± 3.98	6.15 ± 8.68	> 0.05
CD4, CD8	4.58 ± 2.62	4.96 ± 3.44	3.02 ± 1.62	> 0.05

IM: Immunonutrition; NN: Normal nutrition; SE: Standard enteral nutrition.

were recorded.

The Graph Pad Prisma V.3 software package was used for statistical evaluations. For data analysis, in addition to descriptive statistics (mean ± SD), the Friedman test for repeated multiple measures, the Kruskal-Wallis test for independent multiple group comparisons, Dunn's multiple comparison test for sub-group comparisons, the Wilcoxon test for paired group comparisons, and Fisher and χ^2 tests for repeated measures of qualitative data were used. A *P* value < 0.05 at the 95% confidence interval was considered significant.

RESULTS

A total of 14 patients were excluded from the study due to following reasons: gastrointestinal bleeding that started after the nutrition programme, emergency surgery to relieve obstruction, and uncontrolled blood sugar levels. A total of 42 of patients completed the study. There were no significant differences between nutrition groups with regard to age, gender, albumin, prealbumin, lymphocyte count, and body mass index (BMI) values (Table 2).

At baseline, prealbumin levels were low in all groups, with no significant between-group differences (Table 3). Repeated measurements showed a significant increase only in the IMN group compared to baseline (15.98 ± 8.66, $P = 0.037$).

The number of lymphocytes with a CD4+ surface marker was higher in patients receiving SE nutrition before nutritional support (Table 4), but the difference was not significant. Within and between-group comparisons at the end of nutrition showed no significant differences between groups with regard to CD4+ lymphocyte counts (Table 5).

In patients that received normal nutrition (NN) and SE, the number of CD8+ cells was higher compared to normal counts (Table 5). Also, in patients who received NN, the proportion of CD8+ lymphocytes was significantly higher compared to IMN ($P < 0.05$). Following the nutrition, although a slight increase above the normal values was observed in IMN patients, the difference was not significant compared to baseline and other groups ($P > 0.05$).

CD4+/CD8+ ratio was within the normal range

Table 6 Data on the operation and postoperative follow-up (mean ± SD)

	IM $n = 13$	NN $n = 9$	SE $n = 11$	P
Duration of operation (min)	303.8 ± 160.1	248.9 ± 87.67	288.6 ± 71.28	> 0.05
Blood transfusion (U)	2.84 ± 2.85	3 ± 2.20	2.54 ± 1.80	> 0.05
Wound infection	5	3	2	> 0.05
Pneumonia	2	1	4	> 0.05
Urinary infection	-	-	1	> 0.05
Sepsis	-	-	1	> 0.05
Non-infectious complications	5	3	2	> 0.05
Duration of hospital stay (d)	16.54 ± 14.83	12 ± 3.69	14.22 ± 9.12	> 0.05

IM: Immunonutrition; NN: Normal nutrition; SE: Standard enteral nutrition.

before the nutrition (0.8%-1.8%) in all groups. There was a non-significant increase in that parameter in all groups after nutrition.

CD16+/56+ cell counts were below normal in all groups before and after the nutrition period, with no significant differences compared to baseline in any group. Similarly, there were no significant differences in the proportion of T cells with CD4+, CD8+ (double positive) surface markers before and after the nutrition in any group. Also, groups were similar with regard to these parameters at both time points.

CD69+ counts did not differ between or within the groups before and after the nutrition. In IM group, CD 69 counts after nutrition were obtained higher than the before nutrition group, however it was not statistically significant. Also, when the proportional increase in CD69 surface marker in response to a non-specific immune stimulator such as phytohemaglutinin (PHA) is considered, a significantly higher increase was observed in IM group compared to NN group and SE group ($P < 0.05$).

Of the 42 patients completing the study, 33 underwent radical surgical procedures. Among these patients, there were also no intergroup differences with regard to the duration of operation, incidence of infection, and length of hospital stay ($P > 0.05$) (Table 6).

DISCUSSION

Initially, nutritional support was aimed at meeting the energy needs and providing proteins and other essential micronutrients in order to prevent muscle breakdown and immunosuppression, while now it is more directed at modulation of the immune functions[5].

Among the nutrients with a suggested positive effect on immune functions, arginine, glutamine, fatty acids, and nucleotides have been studied most extensively. The two products that have been studied most are IMPACT, which contains arginine, fatty acids, and nucleotides, and IMMUNE-AID, which contains glutamine in addition[5]. In our study IMPACT was used.

Arginine stimulates T lymphocytes, provides a substrate for nitric oxide, and increases the secretion of insulin and growth hormone. Nitric oxide enhances the splanchnic micro-perfusion *via* vasodilator effects. Ornithine and proline are synthesized from arginine. Polyamine, an important factor for cell division, is synthesized from ornithine. Again, arginine causes volume increase in the thymus, and enhances the functions of macrophages and NK cells. It also accelerates wound healing[7,8,19-22].

Omega 3 fatty acids are the precursors for eicosanoids which include prostaglandin, prostacyclin, thromboxane and leukotrienes. They have anti-inflammatory properties which act through three different mechanisms[8,23].

Nucleotides are the precursors for RNA and DNA and are believed to enhance protein synthesis and T-cell functions[9,24]. Addition of nucleotides in nutrients has led to decreased incidence of fungal infections[25].

T lymphocyte activation, interferon γ, NK cell, immunoglobulin M, phagocytic capacity of leukocytes and increase in the number of lymphocytes have commonly been used by the investigators for the assessment of immune functions[5,26-29]. In the present study we examined certain lymphocyte subpopulations such as CD4+ (T helper), CD8+ (T cytotoxic, suppressor), CD16+/56+ (Natural killer), and CD69 cells.

The value of preoperative immunonutrition has now gained widespread acceptance. A shift from the production of acute phase proteins to building-block protein production, effective control of the immune disorder during the early postoperative period, and improved intestinal micro perfusion and oxygen metabolism have been reported in patients receiving immunonutrition[13,30]. At 2001 Consensus Meeting, a 5 to 10 d period was recommended for preoperative immunonutrition[31]. Thus, in this study nutrition was given for 7 d.

The suppression of immunity has been proposed to affect the prognosis adversely in cancer patients by increasing the growth and metastatic potential of residual tumor cells[32]. It can be expected that the preoperative nutrition may attenuate this suppression.

The patient group(s) most likely to benefit from immunonutrition has not been defined yet. Immunonutrition has been proposed to provide benefits in patients undergoing elective surgery for GIS tumors, while no benefit has been reported for intensive care patients, and increased mortality has been reported in patients with sepsis[12,33,34]. Generally, malnourished patients are accepted as candidates for artificial nutrition. On the other hand, Braga *et al*[15] recommend the use of perioperative immunonutrition in all patient groups regardless of the nutritional status. Our patient group consisted of subjects with moderate or severe malnutrition compared to SGA.

Despite continuing controversy, many studies have been examining the role of immunonutrition in patients with upper GIS tumors, trauma, or in ICU patients. Usually a lower incidence of infections and shortened length of hospital stay were observed in patients with

upper GIS tumors and in ICU patients[5].

Braga et al. found that in the first postoperative day, standard diet and immunonutrition did not differ significantly in their effects on phagocytic capacity, cytokine profile, immunoglobulin levels, number of active T and B lymphocytes, and lymphocyte mitogenicity. However, from the 4th postoperative day, an improvement in immunodepression was observed. In patients receiving normal nutrition preoperatively for whom a significant level of immunosuppression is expected, preoperative immunonutrition has been shown to decrease the postoperative infection rate[15]. Matsuda et al. observed an improved TH1/TH2 balance with the use of pre- and postoperative immunonutrition[34].

Most of the patients with cancer suffer from protein-energy malnutrition. The prealbumin levels were low in every 3 patient groups. After nutrition there was a significant increase only in the immunnutrition group. However, Riso et. al declared that there was no increase in the prealbumin levels between postoperative immunonutrition and control patients[35].

The association between major surgery and the decrease in total number of T lymphocytes, suppressor or cytotoxic T lymphocytes and NK cells has been recognized since 1975[36]. In the present study, in contrast with many other studies, preoperative assessment of immune functions allowed us to examine the effect of immunonutrition on immunosuppression caused exclusively by the tumor and malnutrition. No effect on CD4+ and CD8+ cells was observed, although a partial improvement was observed in the IMN group.

Before nutrition, the number of natural killer cells (CD16+/56+) were below the normal range, and none of the nutritional models caused a significant increase in this parameter. CD69+ counts did not differ significantly between the groups.

In the present study, cellular immune response was assessed only by peripheral blood measurements shortly after the termination of nutrition on day 8. On the other hand, Sakurai *et al*[37] (in non-surgical patients) administered enteral immunonutrition to their patients for 12 wk with positive results. In our opinion, such a delay is not acceptable for patients who will undergo cancer surgery.

In conclusion, in the present study preoperative immunonutrition provided a significant increase in prealbumin levels, but did not alter the T lymphocyte subpopulation counts significantly. Further studies are warranted for the assessment of the effect of immunonutrition on antitumor immune response, and we believe that evaluation of tumor infiltrating cells in addition to peripheral blood parameters may provide new insights on this issue.

COMMENTS

Background

Malnutrition is known to have adverse effects on immunity, such as atrophy in lymph nodes, decrease in lymphocyte count and IgA secretion, and suppression in immunity. It is reported in several studies that nutritional support is useful in patients with malnutrition. With addition of immunonutrient products to nutritional therapies, their beneficial effect on immune response is searched for in many prospective clinical studies. Most of the studies are about postoperative complications, frequency of development of infection, hospital stay, wound healing, weight gain, cost, and mortality. The basic defensive response developed by the host against tumors is directed by cellular immunity. There are few studies detecting the changes in cellular immune response after immunonutrition and before the patient has had the surgical trauma. By evaluating the effect of immunonutrition on immunologic parameters preoperatively, the effects of the inflammatory process produced by surgical stress on the results can be avoided.

Research frontiers

The advantages of the nutritional support can be evaluated by the operative mortality, morbidity, and hospital stay. However, how and with which mechanisms this useful effect develops must be detected separately. For that we must see the nutritional subparameters, albumin, prealbumin, immuneglobulin, and immune parameters such as lymphocyte count, change in T lymphocytes, and NK cells.

Innovations and breakthroughs

The authors emphasized changes in T lymphocytes responsible for cellular immunity with immunonutrition before the operation, and measurement of other parameters responsible for immune response, such as tumor infiltrating lymphocytes.

Application

Immune parameters can also be evaluated when the patients are prepared for operations and patients can be supported with immunonutrition if found deprived.

Peer review

This study is a straightforward analysis of the effects of immunonutrition on immune cellular markers and prealbumin on the postoperative course of patients with tumors. Although this is a negative study, it is helpful in avoiding expensive nutritional supplements in those patients requiring cancer surgery.

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BRIEF ARTICLES

Nonalcoholic fatty liver disease in asymptomatic Brazilian adolescents

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Abstract

AIM: To evaluate the prevalence and clinical characteristics of Nonalcoholic fatty liver disease (NAFLD) among asymptomatic Brazilian adolescents.

METHODS: Transversal observational study included asymptomatic adolescents with central obesity from private and public schools in Salvador-Bahia, northeastern Brazil. The children answered a questionnaire that included age, gender, race, and medical history, and were submitted to a complete physical exam and abdominal ultrasound. Biochemical exams included: ALT, AST, GGT, C reactive protein (CRP), fasting glucose, insulin, cholesterol and triglycerides. Criteria for NAFLD included: the presence of steatosis in ultrasound and/or high level of ALT, negative or occasional historic of intake of alcohol ($\leq$ 140 g/wk), negative investigation for hepatitis A, B, C, auto-immune hepatitis, Wilson disease and hemochromatosis.

RESULTS: From October, 2005 to October, 2006, the study included 1801 subjects between 11 and 18 years of age and a mean age of 13.7 ± 2.0 years. One hundred ninety-nine had central obesity. The prevalence of NAFLD was 2.3%, most of whom were male and white. Insulin resistance (IR) was observed in 22.9% of them and had positive correlations with ALT and GGT ($P < 0.05$). Elevated CRP was observed in 6.9% of the cases; however, it was not associated with WC, IR or liver enzymes.

CONCLUSION: The prevalence of NAFLD in Brazilian adolescents was low. The ethnicity may have influence this frequency in the population studied, which had a large proportion of African descendents.

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Key words: Nonalcoholic fatty liver disease; Insulin resistance; Central obesity; Ethnicity; Adolescents

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a clinical-pathological condition of wide spectrum, which includes steatosis and steatohepatitis, that has a potential to advance to cirrhosis and hepatocellular carcinoma[1].

Obesity is the most relevant risk factor for NAFLD in children and several studies have described the prevalence and risk factors of NAFLD in these populations[2,3].

Reports of NAFLD in the child population was described in boys and girls between 10 and 13 years of age who showed elevated aminotransferase levels and variable degrees of steatosis, inflammation and fibrosis

in hepatic biopsies[4]. NAFLD occurrence varies with race/ethnicity and gender[5]. A recent study shows body mass index (BMI) as an independent factor for hepatic fibrosis in children and adolescents with NAFLD[6]. In adults and adolescents, visceral obesity has been considered the main risk factor for the development of hepatic steatosis[7,8]. However, most studies have been done with convenient samples of obese adolescent groups treated in clinics for obesity, which may influence the prevalence of NAFLD in these individuals[9,10].

In Brazil, previous studies estimated that 15% of students are overweight or obese[11,12]; however, the prevalence of NAFLD in this population is not known. The present study aimed to evaluate the prevalence and the clinical characteristics of NAFLD among asymptomatic Brazilian adolescents.

MATERIALS AND METHODS

Study design and population selection

Transversal observational study included asymptomatic adolescents from seven high schools in Salvador, Bahia, Brazil who presented central obesity. Salvador is a city in the northeast of Brazil, where it is relevant to account for the proportion of African descendents.

The study was approved by the Ethics Committee for Research from MCO-Programa de Pós Graduação em Medicina e Saúde-Universidade Federal da Bahia, Brazil.

Inclusion criteria: Age between 11 and 18 years old; abdominal circumference > 75th percentile, according to age and sex[13]; free and pre-explained consent term, signed by the adolescents and their caregivers.

Exclusion criteria: Previous historic or serologic markers for liver diseases (hepatitis A, B, C), autoimmune disease, metabolic diseases such as Wilsons Disease and hemochromatosis and intake of alcohol above 140 g/wk.

Criteria for diagnosis of NAFLD: Presence of steatosis in ultrasound scan and/or high levels of ALT and/or AST; negative or occasional historic alcohol intake (≤ 140 g/wk); negative diagnosis of other liver diseases (A, B and virus, autoimmune hepatitis, metabolic liver disease).

Clinical evaluation

For anthropometric evaluation, a sample of blood was collected and a superior abdomen ultrasound scan was performed during the same session at the nursing of the schools.

Anthropometric measures: waist circumference (WC) was measured at the end of normal expiration in the middle portion between the last rib and the iliac ridge. For evaluation, reference values were adopted that consider normal abdominal circumference to be ≤ 75th percentile and increased abdominal circumference to be > 75th percentile according to age and sex[13].

Participants were weighed while not wearing coats or shoes or carrying any objects. A Filizola balance was used, with a scale resolution of 0.1 kg. Height was measured by a stadiometer with no shoes and hair accessories; the resolution was 0.5 cm. BMI was calculated by dividing weight by height squared. Subjects were considered to be overweight at a BMI between the 85th and 95th percentile and obese at a BMI above the 95th percentile for a given age and sex, based on Cole[14].

All adolescents underwent superior abdomen ultrasound scan (AUS). The AUS was performed by only one medical doctor. The scanner used was an Aloka, model DynaView II, with colored Doppler and a 3.5 MHz drill. The discrepancy of echogenicity between the liver and kidney was considered as a criterion. Hepatic steatosis was graded as mild, moderate or severe, according to the Saverumuttu *et al*[15] classification.

Serological assays

Blood samples were collected by the technical staff from adolescents after a 12 h fast. The samples were analyzed in a referential laboratory for AST, ALT, GGT, C reactive protein (CRP), fasting glucose and insulin. Aminotransferase alterations were considered when AST was > 36 U/L for girls and > 59 U/L for boys, ALT > 52 U/L for girls and >72 U/L for boys, and GGT > 43 for girls and > 73 U/L for boys. High-sensitivity CRP assay was conducted using latex-enhanced nephelometry.

Individuals who presented elevated ALT, AST and GGT levels underwent the following exams: HBsAg, anti-HCV, anti-HAV-IgM, antibodies (anti-nuclear, anti-muscle, anti-LKM, anti-mitochondrial) α_1-antitrypsin, ceruloplasmin and serum copper.

Serum glucose and insulin were used for the calculation of *homeostasis model assessment* (HOMA) = [fast insulin (μIU/mL) × fast glucose (mg/dL)/22.5] (mg/dL = mmol/L × 18.182), considering ≥ 3.16 as a cutoff point to define insulin resistance[16].

The adolescents diagnosed with NAFLD were provided assistance at the NASH Group.

Statistical analyses

The data were analyzed using Statistical Package for Social Science (SPSS) 13.0. Mistake type I was estimated to be 5%. Spearman was used for the correlation between variables and t-test to compare the mean. The data are expressed as percentage, medium and standard deviation.

RESULTS

Between October, 2005 and October, 2006, seven public and private schools participated in the study. This corresponded to 3500 students. Among these students, 1801 (51.5%) adolescents agreed to participate in the study. The present study included 199 individuals who had central obesity (WC > 75 percentile). Twenty-four (12.1%) of them were excluded: one had a history of alcohol consumption above 140 g/wk and 23 had incomplete data.

Among 175 adolescents evaluated, the mean age was 13.7 ± 2.0 years, 54.3% were female and 71.4% (125)

Table 1 Clinical characteristics of 175 asymptomatic adolescents with central obesity second ethnicity (mean ± SD)

Variable	Nonwhite (n = 125)	White (n = 50)
Sex F/M (%)	58.4/41.6	44.0/5.0
Age (yr)	13.74 ± 2.02	13.70 ± 2.00
BMI (kg/m^2)	27.41 ± 3.27	26.64 ± 3.26
WC (cm)	88.09 ± 7.76	88.65 ± 9.26
AST (U/L)	24.11 ± 4.83[a]	26.53 ± 6.77
ALT (U/L)	31.41 ± 7.32	32.96 ± 13.70
GGT (U/L)	22.03 ± 6.68	21.04 ± 11.69

[a]$P < 0.05$ *vs* white.

Table 2 Clinical characteristics of 4 asymptomatic adolescents with NAFLD

Adolescents	Age (yr)	Sex	Elevated enzyme	Steatosis	Class BMI	Ethnicity
1	17	Boy	AST, ALT, GGT	Moderate steatosis	Obese	White
2	15	Boy	No	Mild steatosis	Obese	White
3	12	Boy	No	Mild steatosis	Overweight	White
4	15	Girl	ALT	No	Obese	Nonwhite

were nonwhite. AST level was statistically higher in white adolescents as compared to nonwhite adolescents ($P < 0.05$), as shown in Table 1.

Overweight was observed in 64.0% of studied adolescents with central obesity. There was a positive correlation between BMI and WC values, as shown in Figure 1.

Hepatic steatosis to AUS was observed in 1.7% (3) individuals. All of them were males and white, two were obese and one was overweight. One of the obese adolescents showed moderate steatosis on ultrasound and elevated ALT, AST and GGT. Two of them had mild steatosis on AUS and normal ALT, AST and GGT.

Among the 175 adolescents studied, 2.8% (5) showed elevated aminotransferases. One girl had isolated elevated ALT. One boy had elevated AST, ALT and GGT. All had BMI > 95th percentile. Elevated GGT was observed in 3 cases, two girls and one boy.

According NAFLD criteria (presence of steatosis in ultrasound scan and/or high levels of ALT and/or AST) four (2.3%) adolescents had NAFLD diagnosis (Table 2).

WC had a statistically significant correlation with ALT and GGT ($P < 0.05$). This finding remained after exclusion of a few outliers. IR was observed in 22.9% (40) of adolescents. Only one (1) adolescent with isolated elevated GGT had IR. Insulin resistance also had a statistically significant correlation with ALT and GGT ($P < 0.05$) after exclusion of a few outliers.

Elevated CRP was observed in 6.8% (12) of adolescents. However, adolescents with hepatic steatosis and/or elevated aminotransferases did not have elevated CRP.

DISCUSSION

The present study shows the prevalence and clinical characteristics of NAFLD among Brazilian asymptomatic adolescents.

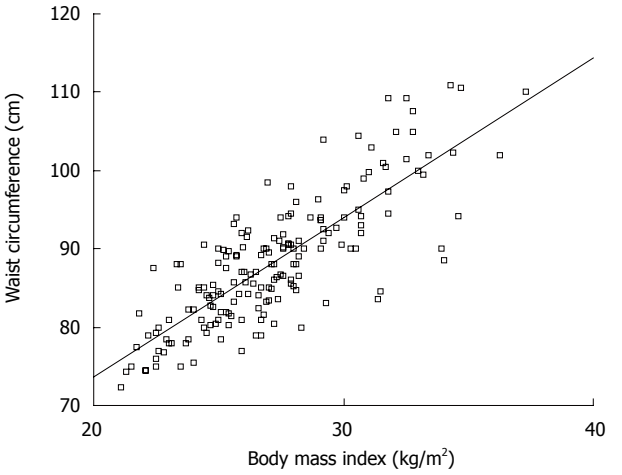

Figure 1 Correlation between body mass index and waist circumference in 175 asymptomatic adolescents with central obesity, Salvador-Bahia, 2005-2006.

The prevalence of 2.3% is consistent with some studies based on the frequency of elevated aminotransferases or fatty liver in obese populations[8,17]. Tominaga *et al*[18] found a prevalence of 2.5% fatty liver in a Japanese study of 810 obese children between 4 and 12 years old, and Alavian *et al*[19] found among 966 obese children in Iran aged 7-18 years 1.8% with elevated ALT.

However, the majority of the cases presented central obesity, identified through isolated measure of WC. It also has been demonstrated as an independent risk factor for development of NAFLD in adults and children, and it is considered more important than obesity[20]. Central obesity has been related to IR, one of the factors related to the pathogenesis of NAFLD[21,22].

Positive correlation between BMI and WC in adolescents, as observed in this study, is also a common finding related to cardiovascular disease in this population[23,24].

This investigation also observed that the majority of adolescents with fatty liver on AUS were obese (BMI ⩾ 95th percentile), which may be related to the degree of association between steatosis and BMI[9,25].

Higher frequency of NAFLD in adolescents has been shown[5,9]; however, other factors may explain this difference between the present investigation and prior ones. The majority of them have included adolescents or children from obesity clinics[26,27]. Another factor was that only half of the eligible children agreed to participate of the study. The discrimination that may occur among obese young people may influence the decision of these people to not consent to the study.

Another influence factor may be ethnicity. It has been considered as an important factor for the different frequencies of NAFLD in children populations. It is more common in Hispanic and less so in black adolescents[5]. In Salvador-Bahia, a city in the northeast of Brazil, the majority of the population are of African descendents. Although this study did not aim to evaluate race, these simple of the population came from an area

in Brazil where a relevant factor is the proportion of African descendents, and it may explain the low prevalence of NAFLD in this study.

The characteristics of disease appear to be similar to those in other studies. Gender has not been considered to be a risk factor for NAFLD in children[28]; however, a higher degree of hepatic steatosis has been found in boys compared to girls, at a ratio of 2:1[5,18]. In this sample, the adolescents with NAFLD were males, corroborating previous reports.

Elevated ALT levels in asymptomatic individuals have been considered useful as a marker to screen patients with NAFLD; they have a correlation with abdominal fat[29], and higher levels of ALT correlated to grades of fat in hepatocytes[3,25]. In obese children, ALT has been considered as a predictor of NAFLD[27]. Elevated levels of ALT were observed only in one adolescent, who had moderate degree of liver steatosis, and in one adolescent with normal AUS, thus showing a correlation between WC and IR.

The frequency of IR was relevant to this study. And despite the positive correlation with ALT and GGT, elevated hepatic enzymes or steatosis were not common. The literature shows that IR is a risk factor for and is associated with NAFLD, mainly with elevated ALT[2,30].

The elevation of CRP in obese adults has been considered a risk factor for progression of NASH[31], and the low prevalence of NAFLD in this study may justify the low occurrence of elevated CRP.

Two female adolescents had isolated elevated GGT. However, GGT is not considered a predictor of NAFLD in obese children. In studies with adults, GGT is associated with NAFLD and the metabolic syndrome, especially in women[32-34]. Fraser *et al*[35], when studying hepatic enzymes in women who had and had not diabetes, observed an association of serum GGT with IR in both groups. We observe that one adolescent with elevated GGT also had IR.

In conclusion, NAFLD in asymptomatic Brazilian adolescents was most frequent among white males with central obesity and the mean age of 13.7 ± 2.0 years. These adolescents came from an area in Brazil where it is relevant to consider the proportion of African descendents, and the influence of ethnicity on the prevalence the NAFLD may be an important factor in this population. However, this hypothesis deserves future study.

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COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) is a common condition among in obese children patients. However, the prevalence of NAFLD has varied in different populations. The influence of gender, risk factors and ethnicity have been discussed. However, more studies are considered necessary to elucidate the relevance of this liver disease in adolescents.

Research frontiers

This study reported the findings of NAFLD prevalence of 2.3% in 175 adolescents with central obesity. The prevalence of NAFLD in these adolescents was lower than others studies and we speculate that ethnicity may influence the prevalence in this population. They come from an area in Brazil where a relevant factor is the proportion of African descendents. However, this hypothesis deserves future study.

Innovations and breakthroughs

This is one of the first Brazilian studies which evaluated the prevalence of NAFLD in adolescents with central obesity. This investigation showed a low prevalence of this liver disease and we hypothesized the influence of ethnicity in the lower frequency NAFLD in this population.

Applications

The prevalence of NAFLD and associated factors in different populations is important for understanding its development, and to implement preventive strategies to control of the disease.

Terminology

NAFLD means Non-alcoholic Fatty Liver Disease; HOMA-IR is homeostasis model assessment index-insulin resistance

Peer review

The work is interesting. Rocha *et al* describe the epidemiology of NAFLD in a cohort of children from Brazil. The study is strengthened by a solid research design and large numbers. While the findings are not particularly novel, it is helpful to have this sort of basic epidemiology data in the literature.

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BRIEF ARTICLES

Analysis of the histologic features in the differential diagnosis of intrahepatic neonatal cholestasis

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Abstract

AIM: To compare the histologic features of the liver in intrahepatic neonatal cholestasis (IHNC) with infectious, genetic-endocrine-metabolic, and idiopathic etiologies.

METHODS: Liver biopsies from 86 infants with IHNC were evaluated. The inclusion criteria consisted of jaundice beginning at 3 mo of age and a hepatic biopsy during the 1st year of life. The following histologic features were evaluated: cholestasis, eosinophilia, giant cells, erythropoiesis, siderosis, portal fibrosis, and the presence of a septum.

RESULTS: Based on the diagnosis, patients were classified into three groups: group 1 (infectious; n = 18), group 2 (genetic-endocrine-metabolic; n = 18), and group 3 (idiopathic; n = 50). There were no significant differences with respect to the following variables: cholestasis, eosinophilia, giant cells, siderosis, portal fibrosis, and presence of a septum. A significant difference was observed with respect to erythropoiesis, which was more severe in group 1 (Fisher's exact test, P = 0.016).

CONCLUSION: A significant difference was observed in IHNC of infectious etiology, in which erythropoiesis was more severe than that in genetic-endocrine-metabolic and idiopathic etiologies, whereas there were no significant differences among cholestasis, eosinophilia, giant cells, siderosis, portal fibrosis, and the presence of a septum.

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Key words: Intrahepatic cholestasis; Liver histopathology; Neonatal jaundice; Neonatal liver disease

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INTRODUCTION

The frequency of cholestatic jaundice is difficult to evaluate with certainty, but varies between 1:2500 and 1:5000 newborns[1-3]. The initial approach in the diagnosis of cholestasis is to distinguish between intrahepatic and extrahepatic causes, as the latter etiology requires early surgical intervention[4]. In general, intrahepatic neonatal cholestasis (IHNC) represents 2/3 of the cases of neonatal cholestasis[5-9]. The most common causes of IHNC are of infectious origin[10-12]. In septicemia, manifestations of hepatic origin represent only one component of the involvement of multiple organs, of which adequate treatment offers the best chance of recovery[13]. Any serious bacterial infections during the neonatal period can result in jaundice[14]; however, there seems to be a more frequent association with urinary tract infections, especially when the pathogen is *E. coli*[15]. In addition, other

infections have been observed, such as syphilis, toxoplasmosis, rubella, and cytomegalovirus (CMV)[16-20].

Despite the many possible etiologies for IHNC[5], 13%-78% of the cases have been reported to be idiopathic[21-24]. Idiopathic IHNC implies that the liver suffers inflammatory alterations of unknown cause, with no evidence of blockage of the biliary tree, and infectious agents or metabolic errors have been ruled out[1,25,26]. There are cases of idiopathic IHNC which are considered spontaneous in which there is familial recurrence, therefore sporadic cases could possibly consist of a viral injury or another environmental factor that affect the transitory form of the immature liver of the newborn; however, the characteristics are similar in both cases[13,27].

Liver biopsy is currently used to confirm the clinical diagnosis and to assess the degree of necroinflammatory injury or fibrosis. Most studies of percutaneous liver biopsy are retrospective analyses and the aim is usually to differentiate biliary atresia from neonatal hepatitis[12,21,28-32].

There are no data available in the literature pertaining to the histologic features present in neonatal hepatitis to aid in the differential diagnosis of IHNC. The objectives of the present study were to analyze and compare the histologic features of the liver in IHNC of infectious, genetic-endocrine-metabolic, and idiopathic etiologies, in the search for features which can facilitate the diagnostic process.

MATERIALS AND METHODS

Eighty-six patients submitted to liver biopsy during IHNC investigation between March 1982 and December 2005, 72 from the State University of Campinas Teaching Hospital (UNICAMP) and 14 from the Children's Institute of the University of São Paulo (USP). Among the 86 hepatic biopsies, 5 were surgical and 81 were percutaneous. The inclusion criteria consisted of jaundice beginning at 3 mo of age and hepatic biopsy during the first year of life.

In order to establish the etiology of IHNC, the following were reviewed: serum alpha-1-antitrypsin, sweat sodium and chloride test, innate metabolic errors in urine, polymerase chain reaction for CMV antigenemia, and serology for CMV, human immunodeficiency virus, hepatitis B virus (HBV), hepatitis C virus (HCV), Epstein-Barr virus, rubella virus, *Toxoplasma gondii*, and *T. pallidum*.

Seven histologic variables were evaluated by means of an investigation protocol: cholestasis, eosinophilia in the inflammatory infiltrate, presence of giant cells, erythropoiesis, siderosis, portal fibrosis and the presence of a septum. Siderosis and cholestasis were evaluated by Perls' staining and portal fibrosis and the presence of a septum by Masson staining.

The histologic variables (cholestasis, eosinophilia in the inflammatory infiltrate, presence of giant cells, erythropoiesis, siderosis and portal fibrosis) were classified according to the degree of intensity using a grading system (Table 1). The histologic variable, septum was classified by: presence or absence.

Ethical aspects

The present research study was approved by the medical research ethics committees of both institutions. Informed consent was not required because liver biopsies were performed during the course of clinical evaluation.

Statistical analysis

In order to verify associations between categorical variables, the χ^2 test was used. When the expected values were < 5, the Fisher's exact test was used[33]. Significance was established as $P \leqslant 0.05$ in all tests. The computer software used was SAS for Windows, version 8.02 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Based on the etiology of IHNC, the patients were classified into three groups: group 1 (infectious; $n = 18$), group 2 (genetic-endocrine-metabolic; $n = 18$), and group 3 (idiopathic; $n = 50$). The etiologies of IHNC are presented in Table 2.

Twenty-seven patients were females and 59 were males. Patients were predominantly boys in all 3 groups ($P = 0.407$).

The mean age at the time of liver biopsy was as follows: group 1, 2 mo and 15 d (range, 1-6 mo and 9 d); group 2, 2 mo and 15 d (range, 1-6 mo and 8 d); and group 3, 2 mo and 24 d (range, 13 d-9 mo and 5 d). There were no statistical differences among the groups ($P = 0.428$).

Table 3 shows values for birth weight, weight during the first medical visit and stature at birth for the groups. There were no significant differences among the groups according to the variables: weight during the first medical visit and stature at birth. However, a significant difference was observed for birth weight, which was lower in group 1 in relation to groups 2 and 3 ($P = 0.014$).

The degree of cholestasis was not significantly different between the 3 groups studied ($P = 0.078$; Figure 1A). The presence of giant cells, graded as absent, mild, moderate, and severe in groups 1, 2, and 3 did not show any significant differences ($P = 0.144$; Figure 1B).

The presence of eosinophils in the inflammatory infiltrate did not differ when the genetic-endocrine-metabolic and/or idiopathic groups were compared ($P = 0.056$; Figure 1C). A significant difference was observed for to the variable, erythropoiesis in group 1 ($P < 0.05$; Figure 1D).

With respect to siderosis, there was no correlation with the etiology of IHNC ($P = 0.973$; Figure 1E).

With respect to progression of IHNC to chronic stages, portal fibrosis (Fisher's exact test $P = 0.86$) and the presence of a septum ($\chi^2 = 3.83$; $P = 0.147$) were not related to the etiology of IHNC (Figure 1F and G).

DISCUSSION

Liver biopsy is recommended for the diagnosis of cholestasis of unknown etiology. The interpretation of a single liver biopsy in a child with neonatal cholestasis is

Table 1 Grading system used for histological parameters: cholestasis, eosinophilia in the inflammatory infiltrate, presence of giant cells, erythropoiesis, siderosis, and portal fibrosis

	Mild	Moderate	Severe
Cholestasis	Biliary pigment deposits in few hepatocytes of zone 3 acini	Hepatocytes with biliary pigment in two zone 3 acini associated with the presence of rare canalicular bilirubinostasis	The majority of the hepatocytes with biliary pigment biliary associated with several canalicular bilirubinostasis
Eosinophilia in the inflammatory infiltrate	Rare eosinophils in few space-porta	Some eosinophils in many space-porta and rare in the parenchyma	Many eosinophils in all the space-porta and several in the parenchyma
Presence of giant cells	Occurring in a maximum of 30% of the hepatocytes	Between 30% and 60% of the hepatocytes	> 60% of the hepatocytes
Erythropoiesis	Rare groupings of erythroblasts	Some groupings of erythroblasts	Groupings of erythroblasts and megakaryocytes
Siderosis	Deposits of ferric pigment in only a few Kupffer cells	Deposits of ferric pigment in Kupffer cells and a few hepatocytes	Deposits of ferric pigment in the majority of Kupffer cells and many hepatocytes
Portal fibrosis	Discrete widening of some space-porta	Widening of some space-porta	Widening of all space-porta

Table 2 Etiologies of intrahepatic neonatal cholestasis

Groups	Etiology	Number of cases
1	Neonatal sepsis	6
	Cytomegalovirus	6
	Urinary tract infection	3
	Syphilis	1
	Toxoplasmosis	2
2	Alpha1-antitripsyn deficiency	2
	Other metabolic diseases	6
	Galactosemia	2
	Alagille Syndrome	2
	Byler's Disease	1
	Cystic Fibrosis	1
	Secondary to use of parenteral nutrition	1
	Down's Sindrome	1
	Panhypopituitarism	2
3	Idiopathic	50
Total		86

Table 3 Clinical characteristics of the patients during the first evaluation, in accordance with groups 1 (infectious), 2 (genetic-endocrine-metabolic) and 3 (idiopathic)

	Group 1	Group 2	Group 3	*P*
Birth weight (g)	2160 (SD = 650)	2780 (SD = 594)	2750 (SD = 767)	0.014
Weight during the first medical visit (g)	3040 (SD = 1014)	3567 (SD = 1170)	3970 (SD = 1074)	0.105
Stature at birth (cm)	44.5 (SD = 4.26)	47 (SD = 3.51)	48 (SD = 5.22)	0.373

A significant difference was observed for birth weight, which was lower in group 1 in relation to groups 2 and 3 ($P < 0.05$).

limited by the dynamics of the disease. Interpretation of the biopsy findings is pathologist-dependent and requires experience[4,33].

Most studies involving percutaneous liver biopsies are retrospective analyses, using the clinical course, surgical or autopsy results as a gold standard. However, the goal is usually to differentiate biliary atresia from neonatal hepatitis[2f,28-32].

Many cholestatic conditions are expressed differently with time. Liver biopsy can also provide disease-specific findings. Examples include PAS-positive granules in alpha-1 antitrypsin deficiency, paucity of ducts in Alagille syndrome, necroinflammatory duct lesions in sclerosing cholangitis, and other findings that are relatively specific for metabolic and storage diseases[4,34,35].

Hepatic erythropoiesis was more evident in INHC of infectious etiology. This was not clear in the idiopathic group, which was likely due to the participation of an undetected infectious agent. On the other hand, hepatic erythropoiesis strongly suggests an infection.

Fetal hematopoiesis has an onset in the beginning of gestation. Initially, hematopoiesis is restricted to the vitelline bag up to the gestational age of 6-8 wk, when the liver starts to be the predominant organ of production. At 20 wk, the bone marrow becomes the main organ of hematopoiesis and remains as the primary reservoir for the circulating population of immune cells. However, when there are infectious processes, hepatic production persists[34].

The presence of eosinophilia in the inflammatory infiltrate, an important marker of neonatal hepatitis[35], did not show significant differences when compared to the genetic-endocrine-metabolic and/or idiopathic groups. The degree of cholestasis, a morphologic variable, which is extremely important for the differential diagnosis of extra- and intra-hepatic cholestasis, did not show a significant difference between the 3 patient groups. Similar findings were observed in relation to progression to chronic stages, demonstrating that portal fibrosis or a septum is not related to the etiology of the process. In relation to siderosis and the presence of giant cells, our findings did not demonstrate any correlation with the etiology of IHNC.

In conclusion, there are no data available in the literature analyzing the histologic features usually present in neonatal hepatitis in the differential diagnosis of IHNC. There were no significant differences among different etiologies of IHNC in relation to the following histologic features: cholestasis, eosinophilia, giant cells, portal fibrosis, the presence or absence of a septum, and siderosis. A significant difference was observed in IHNC of infectious etiology, which presented with more severe

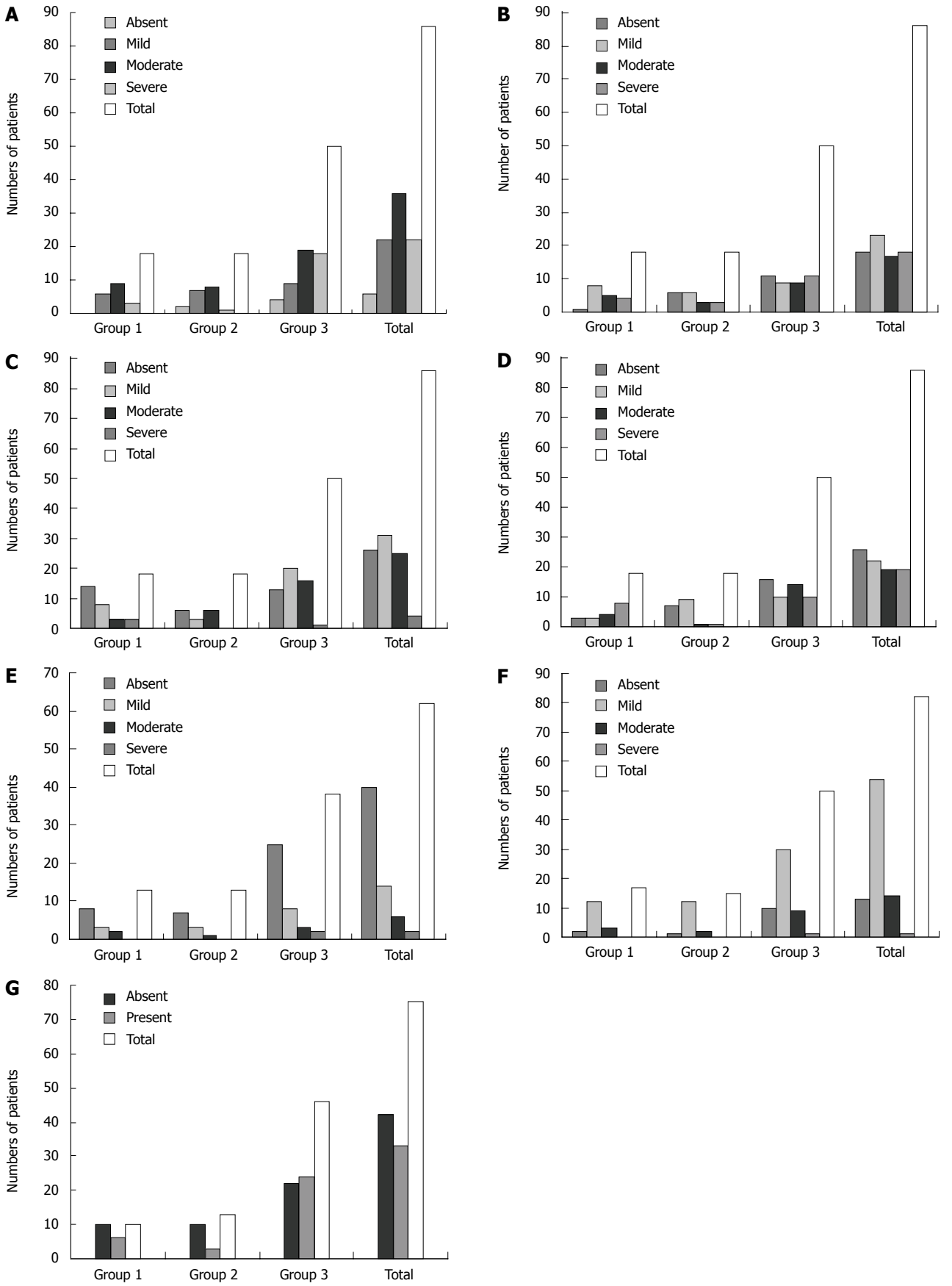

Figure 1 Results of analysis of histologic features present in intrahepatic neonatal cholestasis (IHNC). A: Cholestasis: there was no significant difference between the 3 groups studied ($P > 0.05$); B: Giant cells: there was no significant difference between the 3 groups studied ($P > 0.05$); C: Eosinophils: there was no significant difference between the 3 groups studied ($P > 0.05$); D: Erythropoiesis: there was a significant difference in group 1 ($P < 0.05$); E: Siderosis: there was no significant difference between the 3 groups studied ($P > 0.05$); F: Portal fibrosis (graded as absent, mild, moderate, and severe): there was no significant difference between the 3 groups studied ($P > 0.05$); G: Septum (graded as absent or present) in groups 1 (infectious), 2 (genetic-endocrine-metabolic) and 3 (idiopathic): there was no significant difference between the 3 groups studied ($P > 0.05$).

erythropoiesis than the genetic-endocrine-metabolic and idiopathic etiologies.

In the present study, erythropoiesis was more severe in cases of infectious etiology than in genetic-endocrine-metabolic and idiopathic etiologies and should prompt an investigation for infection.

COMMENTS

Background

Seven histologic variables were evaluated in 86 patients submitted to liver biopsy during intrahepatic neonatal cholestasis (IHNC) investigation: cholestasis, eosinophilia in the inflammatory infiltrate, presence of giant cells, erythropoiesis, siderosis, portal fibrosis and the presence of a septum. There were no significant differences among the different etiologies of IHNC in relation to: cholestasis, eosinophilia, giant cells, portal fibrosis, the presence or absence of a septum, and siderosis. A significant difference was observed in IHNC of infectious etiology, which presented with more severe erythropoiesis than the genetic-endocrine-metabolic and idiopathic etiologies and should prompt an investigation for infection.

Research frontiers

Because of the several possible diagnoses with similar clinical presentation we need to look for features that can help in the diagnostic process of IHNC and a scoring system for histologic features could be useful.

Innovations and breakthroughs

The initial approach of liver biopsy is usually used to distinguish between intrahepatic and extrahepatic neonatal cholestasis. This is the first study to analyze standardized histologic features usually present in IHNC (cholestasis, eosinophilia in the inflammatory infiltrate, presence of giant cells, erythropoiesis, siderosis, portal fibrosis and the presence of a septum) in the differential diagnosis of IHNC.

Applications

Liver biopsy should be interpreted by a pathologist with expertise in pediatric liver disease.

Terminology

Cholestasis, eosinophilia in the inflammatory infiltrate, presence of giant cells, erythropoiesis, siderosis, portal fibrosis and the presence of a septum are histologic features usually present in neonatal hepatitis. A scoring system for these histologic features was used in the differential diagnosis of IHNC.

Peer review

This is a well written analysis of the histologic features in the differential diagnosis of IHNC. Although it is not a landmark finding the report is of value in that it points out that severe erythropoiesis would be a criterion indicating IHNC of infectious etiology.

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BRIEF ARTICLES

Application of Stool-PCR test for diagnosis of *Helicobacter pylori* infection in children

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Abstract

AIM: To evaluate the usefulness of stool-PCR test for diagnosis of *Helicobacter pylori* (*H pylori*) infection in pediatric populations.

METHODS: Based on endoscopic features (including nodular gastritis, erosive duodenitis and ulcer) and/or a positive rapid urease test (RUT) obtained during endoscopy, 28 children from a group of children admitted to the Children's Medical Center of Tehran for persistent upper gastrointestinal problems were selected to compare biopsy-based tests with stool-PCR. Their gastric activity and bacterial density were graded by the updated Sydney system, and their first stool after endoscopy was stored at -70℃. Biopsies were cultured on modified campy-blood agar plates and identified by gram-staining, biochemical tests, and PCR. Two methods of phenol-chloroform and boiling were used for DNA extraction from *H pylori* isolates. Isolation of DNA from stool was performed using a stool DNA extraction kit (Bioneer Inc, Korea). PCR was performed using primers for detection of *vacA*, *cagA*, and *16srRNA* genes in both isolates and stool.

RESULTS: Sixteen out of 28 child patients (57%) were classified as *H pylori* positive by biopsy-based tests, of which 11 (39%) were also positive by stool-PCR. Sensitivity and specificity of stool-PCR was 62.5% and 92.3% respectively. *H pylori* was observed in histological sections for 10 out of 11 stool-positive patients. Association was observed between higher score of *H pylori* in histology and positivity of stool-PCR. Also association was observed between the more severe form of gastritis and a positive stool-PCR.

CONCLUSION: Association between higher score of *H pylori* in histology and a positive stool-PCR make it a very useful test for detection of *H pylori* active infection in children. We also suggest that a simple stool-PCR method can be a useful test for detection of *H pylori* virulence genes in stool.

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Key words: *Helicobacter pylori*; Non-invasive diagnosis; Stool-PCR; Histology; Score; Children; Iran

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INTRODUCTION

Helicobacter pylori (*H pylori*) infection in humans is associated with gastritis, gastric ulcer, and gastric cancers[1,2]. Infection occurs mainly in childhood and infected individuals usually carry it for life unless treated[3,4]. Epidemiology of infection by *H pylori* has been characterized by a linear increase with age in western industrial countries and by a large number of children and juveniles being infected in developing countries[5]. Currently used methods for diagnosis of *H pylori* infection, such as culture, histology, and rapid urease test (RUT) are very sensitive and highly specific tests, but require invasive sampling. The non-invasive methods, such as serology and urea breath test (UBT), are also sensitive and specific; however, positive results obtained by serology do not necessarily indicate current infection by *H pylori*[6,7]. UBT requires an expensive instrument, which is not always available in routine clinical laboratories, especially in developing countries. In addition the performance of the test has been associated with

some disadvantages for infants and very young children, as well as patients with certain neurological disorders[6,7]. *H pylori* is not an intestinal pathogen, and therefore is expected to be present in low concentrations in stool; however, it can be detected in stool specimens by *H pylori* stool-antigen (HpSA) test, PCR, or even culture[8-12]. The HpSA test has been shown to be very useful, especially in children; however, various commercial tests have shown some discrepancies in different geographical areas[13-15]. Stool-culture is a very specific method; however, the massive numbers of diverse micro-organisms in stool makes it very difficult in routine practice[8,12]. Stool-PCR may also be a very useful method in detection of *H pylori* infection, but reported success rates for the detection of *H pylori* DNA in feces vary from 25% to 100%[6,8]. This variability is probably due to *H pylori* degradation in the gastrointestinal tract and/or the presence of inhibitors such as complex polysaccharides[16,17]. The purpose of this study was to evaluate the usefulness of the stool-PCR test for diagnosis of *H pylori* infection in pediatric populations.

MATERIALS AND METHODS

Patients

Based on endoscopic features (including nodular gastritis, erosive duodenitis, or ulcers) and/or a positive rapid urease test obtained during endoscopy, 28 children from a group of children admitted to a children's medical center in Tehran for persistent upper gastrointestinal problems were selected to compare biopsy-based tests and stool-PCR. Of these patients, two antral biopsies similar to that of RUT were obtained for culture and histology, and the first stool after endoscopy but before antibiotic therapy was collected and stored at -70℃. These children were asked to have a vegetable free diet 24 h before sampling. Stool samples were also collected from a few healthy children that showed no symptoms. Patients who tested positive by culture or positive by both RUT and histology were considered as positive controls and those who tested negative by all three endoscopy-based tests were considered as negative ones.

Biopsy-based tests

Culture of biopsy samples was performed as previously described[12,18]. Briefly, antral biopsies were placed in a modified campy-thio medium and incubated at 37℃ under a micro-aerobic atmosphere. After 3 d, 20 μL of the enrichment culture was streaked onto modified campy-blood agar and incubated for 5-10 d until colonies were evident. The grown colonies were identified by gram-staining, oxidase, urease, and nitrate-reduction tests.

RUT was performed using urea broth as previously described. The RUT result was read either within 2 h at endoscopy room or after overnight incubation under a micro-aerobic atmosphere at 37℃ according to the previously described protocol[12,18]. Histological examination of the biopsies was performed after H&E, and Geimsa staining *H pylori* density, gastritis, and inflammation were graded according to the modified Sydney system[19,20]. The cases of gastritis with follicular formation were classified as follicular gastritis either with or without activity[20].

DNA extraction and PCR

Two methods of phenol-chloroform and boiling were used for DNA extraction from *H pylori* isolates. For the first one, a pool of colonies in 2 mL sterile 0.9% NaCL, was centrifuged at 10000 g, the pellet was resuspended in 400 μL of extraction buffer (10 mmol/L Tris-HCL, pH 8.0; 5 mmol/L EDTA, 0.1% sodium dodecyl sulfate), and proteinase K at final concentration of 0.5 mg/mL was added to homogenizates. Samples were incubated at 55℃ for 2-4 h before incubation at 95℃ for 10 min. DNA was purified by phenol-chloroform, precipitated by absolute ethanol at -20℃ in presence of 0.3 mol/L sodium acetate, pelleted by centrifugation at 12000 g for 30 min and allowed to dry in air. The pellet in sterile double-distilled water was quantified by measuring the optical density at 260 nm and stored at -20℃ until they were used as PCR templates. For the second method, a loopful of colonies was suspended in 1 mL of phosphate buffer saline (PBS, pH 7.6), washed by centrifugation at 14000 g for 2 min, and resuspended in 50 μL of sterile, double distilled water. Tubes were then boiled at 95℃ for five minutes and 2 μL of 1/5 dilution of this extract (containing approximately 20 ng of DNA) was immediately used as template for PCR. Isolation of DNA from stool was performed using a stool DNA extraction kit (Bioneer Inc, Korea), where substances inhibiting PCR were removed by filtration according to the manufacturer's instructions. Stool-PCR controls were 3 uninfected feces from the *H pylori*-negative patient (as determined by endoscopy-based tests) seeded or not seeded with known concentrations (equivalent to McFarland No. 5) of 26695 *H pylori* ATCC strain.

PCR primers (Faza Biotech Inc, Iran) were designed on the basis of published sequences of *H pylori 16SrRNA, vacA*, and *cagA*[8,21]. Table 1 resumes the sequences and experimental details for PCR.

RESULTS

The H pylori status

Sixteen out of 28 child patients (57%) were classified as *H pylori* positive by biopsy-based tests. Of 16 *H pylori* positive children 6 were positive by culture, 5 were positive by all of the 3 tests, and 5 were positive by RUT plus histology.

PCR results

DNA isolated from culture positive controls showed amplification for *H pylori* specific primer(s) including *vacA* (*s*, m), *cagA*, and *16srRNA*. Stool-PCR positive controls, which were 3 uninfected feces from the *H pylori*-negative patient containing known concentrations of 26695 *H pylori* ATCC strain, showed amplification for *H pylori* DNA only after purification by column chromatography procedure. No amplification was observed for the negative stool-PCR controls (stool

Table 1 Primers sequences and PCR conditions

Primers	Sequences	Product size (bp)	PCR conditions
16sRNA	5'GCTAAGAGATCAGCCTATGTCC3'	500	95℃ 5 min (1 cycle); 94℃ for 1 min, 55℃ for 1 min
	5'TGGCAATCAGCGTCAGGTAATG3'		72℃ for 2 min (39 cycles); 72℃ for 7 min
VacA (s)	5'ATGGAAATACAACAAACACAC3'	s1: 259	95℃ 4 min (1 cycle); 95℃ for 1 min, 52℃ for 1 min
	5'CTGCTTGAATGCGCCAAAC3'	s2: 286	72℃ for 1 min (35 cycles); 72℃ for 10 min
vacA (m)	5'CAATCTGTCCAATCAAGCGAG34	m1: 570	95℃ 4 min (1 cycle); 95℃ for 1 min, 52℃ for 1 min
	5'GCGTCTAAATAATTCCAAGG3'	m2: 642	72℃ for 1 min (35 cycles); 72℃ for 10 min
cagA	5'AATACACCAACGCCTCCA3'	400	94℃ for 4 min (1 cycle); 94℃ for 1 min, 59℃ for 1 min
	5'TTGTTGCCGCTTTTGCTCTC3'		72℃ for 1 min (35 cycles); 72℃ for 10 min

Table 2 Comparison between the results of biopsy-based tests and Stool-PCR

n /Status	Culture	RUT	Histology	Stool-PCR
1/negative	Negative	Nd	Negative	Negative[a]
2/negative	Negative	Nd	Negative	Negative[a]
3/negative	Negative	Nd	Negative	Negative[a]
4/negative	Negative	Negative	Negative	Negative[a]
5/negative	Negative	Negative	Negative	Negative[a]
6/positive	Positive	Negative	Negative	Negative[b]
7/negative	Negative	Negative	Negative	Negative[a]
8/positive	Negative	Positive	Positive	Positive[c]
9/negative	Negative	Positive	Negative	Negative[a]
10/negative	Negative	Negative	Negative	Negative[a]
11/positive	Positive	Positive	Negative	Negative[b]
12/positive	Positive	Positive	Positive	Positive[c]
13/positive	Positive	Positive	Negative	Negative[b]
14/positive	Negative	Positive	Positive	Positive[c]
15/positive	Positive	Positive	Positive	Positive[c]
16/positive	Positive	Positive	Positive	Positive[c]
17/positive	Positive	Positive	Positive	Positive[c]
18/positive	Positive	Positive	Positive	Positive[c]
19/negative	Negative	Negative	Negative	Negative[a]
20/positive	Negative	Positive	Positive	Positive[c]
21/negative	Negative	Negative	Negative	Negative[a]
22/negative	Negative	Negative	Negative	Negative[a]
23/positive	Positive	Positive	Negative	Negative[b]
24/positive	Positive	Positive	Negative	Negative[b]
25/positive	Positive	Positive	Negative	Positive[c]
26/positive	Negative	Positive	Positive	Positive[c]
27/negative	Negative	Positive	Negative	Positive[d]
28/positive	Negative	Positive	Positive	Negative[b]

Nd: Not-determined; a: True negative; b: False negative; c: True positive; d: False positive. Sensitivity: c/c + b = 62.5; Specificity: a/a + d = 92.3%.

Table 3 Comparison of detected genes in DNA from isolates and DNA from stool

n /Status	Detected genes in					
	Isolate			Stool		
	16sRNA	*vacA*	*cagA*	*16sRNA*	*vacA*	*cagA*
1/negative	-	-	-	-	-	-
2/negative	-	-	-	-	-	-
3/negative	-	-	-	-	-	-
4/negative	-	-	-	-	-	-
5/negative	-	-	-	-	-	-
6/positive[a]	+	+	-	-	-	-
7/negative	-	-	-	-	-	-
8/positive[b,c]	-	-	-	-	-	+
9/negative[b]	-	-	-	-	-	-
10/negative	-	-	-	-	-	-
11/positive[a,b]	-	+	+	-	-	-
12/positive[a,b,c]	-	-	+	-	+	-
13/positive[a,b]	+	-	-	-	-	-
14/positive[b,c]	-	-	-	-	+	-
15/positive[a,b,c]	-	+	-	-	+	-
16/positive[a,b,c]	+	+	+	+	+	-
17/positive[a,b,c]	-	-	+	+	+	-
18/positive[a,b,c]	-	-	+	+	+	-
19/negative	-	-	-	-	-	-
20/positive[b,c]	-	-	-	+	+	-
21/negative	-	-	-	-	-	-
22/negative	-	-	-	-	-	-
23/positive[a,b]	-	-	+	-	-	-
24/positive[a,b]	-	-	+	-	-	-
25/positive[a,b]	-	-	+	-	+	-
26/positive[b,c]	-	-	-	-	+	-
27/negative[b]	-	-	-	+	-	-
28/positive[b,c]	-	-	-	-	-	-

a: Culture positive; b: RUT positive; c: Histology positive.

specimens from *H pylori*-negative patients), even after purification procedure. Eleven biopsied children showed positive stool-PCR of which 10 were positive by biopsy-based tests (Table 2). Sensitivity and specificity of stool-PCR were 62.5% and 92.3% respectively.

In this work, detection of *H pylori* specific virulence genes in both isolates and stool (Table 3) was compared. Also, association between endoscopic features, pathology, score of bacteria, and a positive stool-PCR was studied (Table 4). *H pylori* was observed in histological sections of 10 out of 11 stool-positive patients and association was observed between higher score of *H pylori* in histology and a positive stool-PCR.

DISCUSSION

In our previous study[12], we successfully cultured *H pylori* from stool; however, the sensitivity of stool-culture was low. Using PCR, we detected *H pylori* specific genes in isolates and stool in sick and healthy children. However, when fecal extracts were not subjected to column chromatography, there were no results even for the positive controls. This suggests that the method of DNA extraction used in this work efficiently removed the PCR inhibitors. Various methods has been used for the removing of inhibitors or for the purification of DNA

Table 4 Relationship between endoscopic features of patients, histopathology, score of *H pylori* and detection of DNA in stool

n/Status	Endoscopic feature	Histopa-thology	Score of *H pylori*	Stool PCR
1/negative	Non-ulcer	NSPC	0	Negative
2/negative	Non-ulcer	NST	0	Negative
3/negative	Non-ulcer	Mild chronic gastritis	0	Negative
4/negative	Non-ulcer	Follicular gastritis	0	Negative
5/negative	Non-ulcer	Follicular gastritis + activity	0	Negative
6/positive	Non-ulcer	NSPC	0	Negative
7/negative	Non-ulcer	Mild chronic gastritis	0	Negative
8/positive	Non-ulcer	Follicular gastritis	4	Positive
9/negative	Ulcer	NST	0	Negative
10/negative	Non-ulcer	NSPC	0	Negative
11/positive	Non-ulcer	Follicular gastritis	0	Negative
12/positive	Non-ulcer	Follicular gastritis + activity	4	Positive
13/positive	Non-ulcer	Follicular gastritis	0	Negative
14/positive	Non-ulcer	Moderate chronic gastritis	1	Positive
15/positive	Multiple ulcers	Moderate chronic gastritis	4	Positive
16/positive	Non-ulcer	Moderate chronic gastritis	2	Positive
17/positive	Ulcer	Grading was not possible	1	Positive
18/positive	Non-ulcer	Follicular gastritis + activity	5	Positive
19/positive	Non-ulcer	Mild chronic gastritis	0	Negative
20/positive	Non-ulcer	Follicular gastritis	3	Positive
21/negative	Non-ulcer	NSPC	0	Negative
22/negative	Non-ulcer	NSPC	0	Negative
23/positive	Non-ulcer	Moderate chronic gastritis	0	Negative
24/positive	Non-ulcer	Mild chronic gastritis	0	Negative
25/positive	Non-ulcer	Mild chronic gastritis	0	Positive
26/positive	Non-ulcer	Follicular gastritis	2	Positive
27/negative	Multiple ulcers	Mild chronic gastritis	0	Positive
28/positive	Non-ulcer	Moderate chronic gastritis	3	Negative

NSPC: No significant pathologic change; NST: No suitable tissue.

such as the removal of PCR inhibitors by a polypropylene filter, dilution of fecal suspension, and DNA purification by various biochemical techniques; in many studies with filtration of stool and column chromatography, high sensitivity was observed[8,10-11,14,22-24].

In this work, by detection of various *H pylori* specific genes in stools, 62.5% sensitivity and 92.3% specificity was observed for stool-PCR (Table 2). Nevertheless, by PCR only one or two out of three *H pylori* specific genes were detectable (Table 3). While this permits us to think that the absence of amplification is related to the absence of the detecting gene from the genome or the absence of intact template DNA (in stool), it would be a premature conclusion, since PCR-based absence of an ORF does not necessarily mean its absence from the genome. Also, in a highly recombining genome like *H pylori*, PCR primer annealing sites can pose problems and amplifications may not be generated[25,26]. Thus, we think that for genotyping of *H pylori* from stool, using more than one primer for each gene may enhance detection rate. Many investigators have proposed semi-nested or nested PCR as more sensitive methods for stool-PCR[8,10]. Although these methods reduce background, their disadvantages would be presence of false positive results due to detection of dead bacteria in stool even in low amounts. Sensitivity and specificity of stool-PCR method in this work were acceptable, suggesting that PCR method used in this work was quite adequate for this evaluation.

H pylori is not an intestinal pathogen, and therefore is expected to be present in low concentrations in stool; however, the status of the infection of *H pylori* may influence its density in stool. Thus, we compared histological scoring of *H pylori* with pathological grading and also with the results of stool-PCR. Concordance was observed between higher score of *H pylori* in histological sections and a positive stool-PCR (Table 4). Also, association was observed between the more severe form of gastritis and a positive stool-PCR. Therefore, the degree of stomach colonization by *H pylori* may be important for successful detection of DNA in stool samples. Otherwise, the amount of bacteria excreted in stool may reveal information on the status of *H pylori* infection. Consequently, the association between a higher score of *H pylori* in histology and a positive stool-PCR make it a very useful test for detection of pediatric *H pylori* infection.

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COMMENTS

Background

A reliable non-invasive test for detection of *Helicobacter pylori* (*H pylori*) infection in routine practice is essential, especially for children since the application of biopsy-based tests is more difficult for them. Serological tests do not necessarily indicate active infection by *H pylori*, and urea breath test (UBT) is expensive and not available in routine clinical laboratories, especially in developing countries. The *H pylori* stool-antigen (HpSA) test has been shown to be very useful, especially in children; however, various commercial tests have shown some discrepancies in different geographical areas. Stool-PCR may be a very useful test in specific detection of *H pylori*. In this study, we evaluated the performance of stool-PCR in diagnosis of active infection in children.

Research frontiers

Stool-PCR is a very useful method for detection of *H pylori* genes in stool. It is interesting because *H pylori* specific genes, including virulence genes and the genes involved in its resistance to antibiotics, can be detected by this method. Furthermore, a positive stool-PCR has significance in relation to the status of stomach colonization by *H pylori*.

Innovations and breakthroughs

A stool-PCR method such that used in this work may represent a very specific test for diagnosis of *H pylori* infection. This is the first study to report association between a positive stool-PCR and the degree of stomach colonization, manifested by higher score of *H pylori* in histology.

Applications

A simple PCR method such that used in this work will be quite adequate for detection of *H pylori* infection.

Peer review

In this study, Falsafi *et al*. evaluated the performance of stool-PCR test for diagnosis of current *H pylori* infection in children. The content of the article can be interesting for gastroenterologists who work with the pediatric population, especially with very young children and patients with certain neurological disorders. Stool-PCR may be a very useful method in detection of *H pylori* infection.

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Endoscopic features predictive of gastric cancer in superficial lesions with biopsy-proven high grade intraepithelial neoplasia

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Abstract

AIM: To investigate the macroscopic and clinicopathologic features of gastric cancer in patients with biopsy-suggested high grade intraepithelial neoplasia.

METHODS: Patients with biopsy-confirmed gastric high grade intraepithelial neoplasia were reviewed from January 2001 to March 2008. Pathologic sections were re-evaluated by two senior pathologists. Patients with an *en-bloc* resection of the lesion within two months after the diagnosis of high grade intraepithelial neoplasia were enrolled in the study. Clinical manifestations, endoscopic features, biopsy and surgical pathology of all patients were collected and analyzed. The data acquired were subjected to univariate and multivariate analysis.

RESULTS: Seventy-two superficial gastric lesions with a pathologic diagnosis of high grade intraepithelial neoplasia based on biopsy specimens were enrolled. True high grade intraepithelial neoplasia was finally proved in 16 lesions and gastric cancer in the rest 56 lesions, most of which (96.4%) were differentiated carcinomas. The result of univariate analysis indicated that the size and the presence of marked ulcer plaque or scar in a superficial lesion were independently associated with gastric cancer ($P < 0.05$), when high grade intraepithelial neoplasia was diagnosed by biopsy pathology. The results of multivariate analysis revealed the size greater than 1.5 cm [odds ratio (OR) 18.400, $P < 0.001$] and the presence of 5-odd mm ulcer plaque or scar (OR 10.000, $P = 0.044$) were associated with gastric cancer. Accordingly, the sensitivity, specificity and negative predictive value of multivariate analysis for predicting "true high grade intraepithelial neoplasia" was 87.5%, 89.3% and 96.2%, respectively.

CONCLUSION: Macroscopic findings are of value in differentiation between high grade intraepithelial neoplasia and superficial gastric cancer. This may simplify patient work-up and save costs for patients and healthcare system.

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Key words: Stomach neoplasm; Precancerous conditions; Carcinoma *in situ*; Endoscopy; Gastrointestinal; Pathology

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INTRODUCTION

The reported incidence of severe gastric dysplasia developing into gastric carcinoma varies from 10% to 85% in different studies[1-10]. These studies were mainly performed in the 1980s[2,3] and 1990s[4-7], except for a recent study drawing a significantly different conclusion compared with the other studies[8]. Although these studies contributed to the current understanding of gastric cancer, their results were obtained mainly through

long-term follow-up and re-biopsy. In clinical practice and in our experience, a pathologic diagnosis based on biopsy samples may be inadequate in evaluating the severity of lesions. Besides site and quantity, biopsy bias is inevitably associated with the activity of tissue inflammation and regeneration. For instance, it is well recognized that a biopsy sample containing glandular distortion and atypia on an apparent inflammatory background always leads to a diagnosis of regeneration rather than a diagnosis of neoplasia.

A two-stage evaluation system of intraepithelial neoplasia (IEN) was introduced to gastrointestinal tumors by International Authority for Research on Cancer (IARC) in 2000 to replace the term "dysplasia"[9]. Few relevant studies have been published since then. Our preliminary study on the association between gastric HGIEN and invasive cancer has drawn promising results[10]. Further researches will enhance the clinical understanding of precancerous lesions and help distinguish HGIEN from invasive cancer under routine conventional endoscope, without the aid of complex methodologies like magnifying endoscopy or narrow-band imaging. This would improve the work-up of such patients, potentially saving costs for healthcare system and patients. In the present study, we analyzed the macroscopic and clinicopathologic features of superficial neoplastic lesions in an attempt to identify the macroscopic features independently associated with the presence of cancer.

MATERIALS AND METHODS

Patients

One hundred and four lesions in 103 consecutive patients were pathologically diagnosed as HGIEN of gastric mucosa based on biopsy samples obtained by EGD procedures in Shanghai Ruijin Hospital from January 2001 to March 2008. The charts, EGD reports and histologic sections were reviewed. Histopathologically, suspected stromal invasion was identified in 4 lesions. Endoscopically, lesions with an appearance strongly suggestive of advanced gastric cancer (i.e. Borrmann-type gastric cancer) were found in 21 patients, including fungating type in 1 patient, ulcerated in 7 and infiltrative ulcerated in 13 patients. These patients, excluded from the study, were later proved to have advanced gastric cancer. Seventy-nine superficial lesions were confirmed in 78 patients. Of these 78 patients, 6 refused interventional treatment while the remaining one was not a candidate for resection because of severe co-morbidities and poor overall condition. Seventy-one patients (59 males, 12 females, range 35-82 years, and a mean age of 60.5 ± 9.9 years) with 72 superficial neoplastic lesions were finally enrolled in the present study.

All patients gave their written consent to undergo endoscopic or surgical resection with the knowledge that the lesion may not be carcinomatous or infiltrative and the widely recognized guideline in treating GI neoplastic lesions with a biopsy diagnosis of HGIEN was not well established. Patients for endoscopic treatment were well informed about possibilities of incomplete resection, tumor recurrence, hemorrhage and other complications beforehand.

Endoscopy

EGD procedures were carried out on each patient prior to resection using either EG 410HR or EG 590WR video endoscope (Fujinon, Saitama, Japan). Characteristics of the lesion, including its location, size, contour, and ulceration if present, as well as changes in mucosal rugae and endoscopic type, were documented. If an ulcer or ulcer scar was noted, descriptive terms and an estimation of the size of ulcer plague or scar was obtained. Lesions were classified according to the Paris endoscopic classification of superficial neoplastic lesions[11]. "Superficial" lesions are defined when endoscopic appearance suggests either a small cancer or a noninvasive neoplastic lesion[11]. Recognition of superficial gastric lesions was also based on our experience in the endoscopic diagnosis of early gastric cancer. Biopsy was obtained and all patients were diagnosed as HGIEN based on biopsy samples.

Treatment

Abdominal US and enhanced CT scan were performed and distant metastasis was ruled out in all patients. All patients received *en-bloc* resection within two months after the initial diagnosis of HGIEN. Surgery was preferentially carried out given its advantage in curative resection irrespective of tumor size and invasion depth. Endoscopic resection was strictly limited to type 0- I or 0-Ⅱa lesions with fine deformability and no ulceration. Lesions less than 15 mm in size were subjected to EMR using a strip biopsy method[12] and otherwise to endoscopic submucosal dissection (ESD) using the insulated-tip knife[13].

Pathologic diagnosis

The diagnosis of HGIEN was confirmed by two senior pathologists (Y-B. Z & Q. W). The IARC diagnostic criterion for HGIEN was applied[1], in which HGIEN is characterized by increasing architectural distortion with glandular crowding and prominent cellular atypia. An increased proliferative activity was present throughout the epithelium where no stromal invasion occurred. A lesion suspected of stromal invasion was ruled out of this series. Standardized sectioning of the resected specimen was carried out according to the guideline by JGCA[14]. Photograph of the specimen was taken before sectioning and a schematic map of sectioning was drawn. "True HGIEN" was diagnosed when each section of the *en-bloc* resected specimen met the criteria of HGIEN. Gastric carcinoma is diagnosed when the tumor invades to the lamina propria[1].

Statistical analysis

Endoscopic and clinicopathologic variables that might be predictive of gastric cancer were analyzed, including age, sex, history of melena or weight loss, time frame between the diagnosis of HGIEN and resection, endoscopic type (0- I /0-Ⅱa, 0-Ⅱc, 0-Ⅱa + Ⅱc/0-Ⅱc + Ⅱa, 0-

Table 1 Indications for EGD in 71 patients with biopsy-proven HGIEN of gastric mucosa

Indications for endoscopy	Patients (*n*)
Abdominal pain	39[1]
Abdominal discomfort	10[2]
Abdominal distension	7
Melena	6
Retroxyphoid pain	6
Asymptomatic physical check-up	2
Anemia	1

[1]Four patients also complained of melena and another patient complained of weight loss; [2]One patient also complained of weight loss.

Ⅱc + Ⅲ/0-Ⅲ; mixed type), exact lesion size, location, mucosal rugae changes and presence of marked ulcer plaque or ulcer scar on the lesion. Continuous variables (age, time frame, exact lesion size) were compared using group *t* test when the variables were normally distributed and Wilcoxon test when a normal distribution was not observed. Categorical variables [sex, history of melena or weight loss, endoscopic type (0-Ⅰ/0-Ⅱa, 0-Ⅱc, 0-Ⅱa + Ⅱc/0-Ⅱc + Ⅱa, 0-Ⅱc + Ⅲ/0-Ⅲ; mixed type), lesion size with a cut-off value of 1.5 cm, location, mucosal ruga changes and presence of marked ulcer plaque or ulcer scar] were compared using χ^2 test or continuity-adjusted χ^2 test. Multiple logistic regression analysis was used to identify the factors independently associated with gastric cancer. $P < 0.05$ was considered statistically significant. All analyses were performed using the SAS software (SAS Institute, Cary, NC, USA).

RESULTS

Clinical manifestation

Of the 71 patients, 69 were presented with symptoms while the other 2 were asymptomatic. The time of symptoms ranged 1 wk-11 years. The indications for EGD are presented in Table 1.

Endoscopy and final diagnosis

Of the 71 patients, 69 were proved to have isolated HGIEN lesions in the stomach, one was found to have two biopsy-proven HGIEN lesions both in antrum and in fundus, and the other one was found to have gastric polyposis involving antrum and corpus. Of the 72 lesions, 28 were located in antrum, 20 in angularis, 10 in corpus, 1 in fundus and 11 in cardia. Antrum, angularis and corpus were involved in another 2 lesions.

All patients received either surgical or endoscopic resection within two months of initial diagnosis. The time frame between initial diagnosis of HGIEN and resection was 19.2 ± 11.8 d. Open gastrectomy and laparoscopic-assisted gastrectomy were performed on 53 and 14 patients, respectively. Three patients received EMR and one received ESD. All surgical and endoscopic resections were proved curative with horizontal and vertical R0 margins.

Of the 72 lesions, 16 (22.2%) were pathologically confirmed as true HGIEN of gastric mucosa, 51 (70.8%) were early gastric cancer and 5 (6.9%) were advanced gastric cancer. Among the early gastric cancer, 34 were intramucosal and 17 were submucosal cancer.. Advanced gastric cancer in the 5 cases invaded the superficial layer of muscularis propria. Differentiated adenocarcinoma was found in 54/56 lesions (96.4%) while undifferentiated adenocarcinoma was found in 2/56 lesions (3.6%). A case of true HGIEN of gastric mucosa is presented in Figure 1.

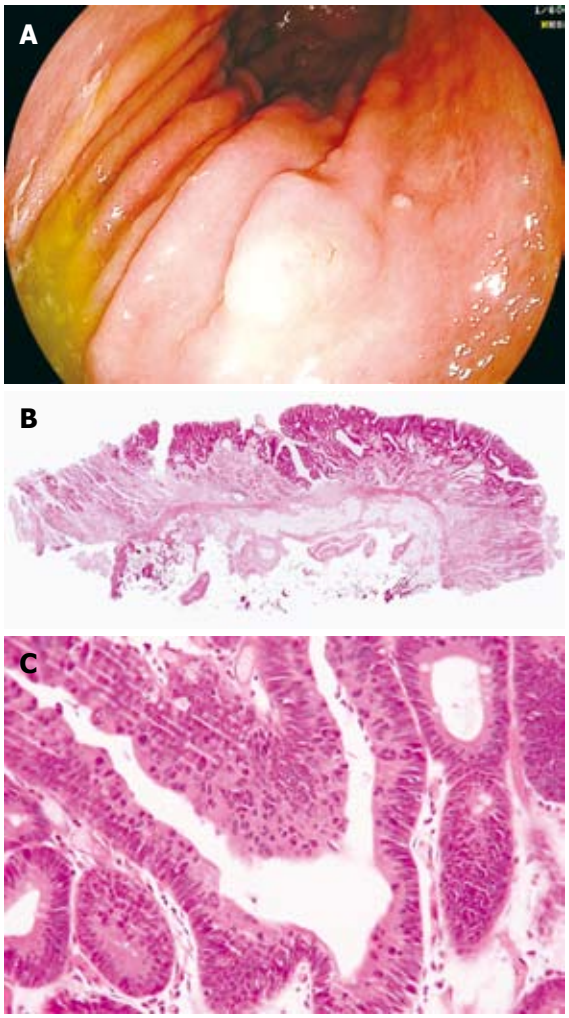

Figure 1 A case of true HGIEN of gastric mucosa. A: Endoscopic view of a sessile polypoid lesion (types 0-Ⅰ) in the greater curvature of gastric corpus. The lesion is approximately 1.2 cm in size with a smooth contour. Biopsy pathology indicated high grade intraepithelial neoplasia; B: Low-power view of the en-bloc resected specimen, showing negative vertical and horizontal margins (HE staining, × 25); C: High-power view shows prominent cellular atypia and increased proliferative activity without stromal invasion, indicating high grade intraepithelial neoplasia (HE staining, × 200).

Differentiation of "true HGIEN" and gastric cancer

All lesions were divided into "true HGIEN" group (*n* = 16) and gastric cancer group (*n* = 56), according to their final diagnosis. A comparison of endoscopic and clinicopathological parameters of the two groups is shown in Table 2.

Twenty-three patients previously underwent EGD procedure within past 2 years. Of the 23 patients, one had normal EGD and 22 were identified to have 23 lesions. Targeted biopsy revealed high grade intraepithelial

Table 2 Comparison between true HGIEN and gastric cancer lesions confirmed by *en-bloc* resection (mean ± SD) *n* (%)

Parameter	True HGIEN group (*n* = 16)	Gastric cancer group (*n* = 56)	*P* value
Age (yr)	60.6 ± 7.5	60.5 ± 10.4	0.987
Male sex	11 (91.7)	49 (87.5)	0.163
History of melena or weight loss	2 (12.5)	10 (17.9)	0.899
History of previous target biopsy	6 (37.5)	17 (30.4)	0.589
Time frame (d)	19.7 ± 11.8	19.0 ± 11.9	0.955
Macroscopic type			
0-Ⅰ/0-Ⅱa	4 (25.0)	9 (16.1)	0.652
0-Ⅱc	9 (56.2)	26 (46.4)	0.488
0-Ⅱa + Ⅱc/Ⅱc + Ⅱa	3 (18.8)	13 (23.2)	0.970
0-Ⅱc + Ⅲ/0-Ⅲ	0 (0)	8 (14.3)	0.249
Mixed type[1]	3 (18.8)	19 (33.9)	0.393
Details of endoscopic findings			
Lesion size (cm)	1.2 ± 0.4	2.6 ± 2.1	< 0.001[2]
> 1.5 cm	2 (12.5)	46 (82.1)	< 0.001[2]
Location (distal stomach)	10 (62.5)	38 (67.8)	0.688
Mucosal ruga changes	0 (0)	5 (8.9)	0.496
Marked ulcer plaque[3] or ulcer scar	1 (6.2)	34 (60.7)	< 0.001[2]

[1]The mixed type included 0-Ⅰs + Ⅱc, 0-Ⅱa + Ⅱc, 0-Ⅱc + Ⅱa and 0-Ⅱc + Ⅲ in this series; [2]*P* < 0.05, Wilcoxon test for continuous variables without a normal distribution and χ^2 test for categorical variables; [3]One or several ulcer plaques/scars at least 5 mm in size.

neoplasia in 17 cases, low-grade intraepithelial neoplasia in 2 and no neoplasia in 4 cases, respectively. Six out of the 23 lesions were finally proved to be true HGIEN of gastric mucosa.

Since endoscopy is an essential method in the diagnosis of gastrointestinal neoplasm and endoscopic appearance of a lesion plays an important role in predicting its characteristics and its invasion depth or metastasis[15], macroscopic findings under conventional endoscopy were analyzed in this study, including macroscopic type, lesion size, location, mucosal ruga changes (e.g. convergence, tapering, abruption, *etc*) and presence of ulcer plaque or ulcer scar, all of which are easily accessible in daily practices. Location was categorized as proximal (body, cardia and fundus of stomach) or distal (antrum and angularis). Presence of marked ulcer plaque/ulcer scar was defined as one or several ulcer plaques/ulcer scars at least 5 mm in size noted on the lesion.

The results of univariate analysis indicate that gastric cancer was associated with the size of lesion and the presence of marked ulcer plaque or ulcer scar ($P < 0.05$). Abruption of mucosal rugae was noted around 5 lesions, all of which were proved to be gastric cancer, so did the 8 lesions presenting with an ulcerated type (0-Ⅱc + Ⅲ or 0-Ⅲ). However, no statistical significance was identified in these parameters.

Multivariate analysis revealed that a lesion larger than 1.5 in size (OR 18.400, 95% CI 3.364-100.653, $P < 0.001$) and the presence of marked ulcer plaque or ulcer scar (OR 10.000, 95% CI 1.062-94.111, $P = 0.044$) were associated with gastric cancer (Table 3). The value of ulcer plaque in the diagnosis of invasive cancer is demonstrated in Figure 2.

A macroscopic criterion for true HGIEN of gastric mucosa was accordingly proposed as a lesion ⩽ 1.5 cm in size, and absence of 5-odd mm ulcer plaque or ulcer scar on the premise that HGIEN was suggested by biopsy pathology. A total 20 lesions in this series met the above criteria, of which, 14 were finally proved to be "true HGIEN" of gastric mucosa, and 6 were proved to be intramucosal gastric cancer. The sensitivity, specificity and negative predictive value of multivariate analysis for predicting "true HGIEN" was 87.5%, 89.3% and 96.2%, respectively.

Table 3 Results of logistic regression analysis of the clinical variables

Variable	Odds ratio (95% CI)	*P* value
Age (yr)	-	0.773
Gender (male)	-	0.328
Endoscopic findings		
0-Ⅱc + Ⅲ/0-Ⅲ	-	0.548
Mixed type[2]	-	0.947
Lesion size >1.5 cm	18.400 (3.364-100.653)	< 0.001[1]
Mucosal ruga changes	-	0.685
Marked ulcer plaque[3] or ulcer scar	10.000 (1.062-94.111)	0.044[1]

[1]*P* < 0.05, multiple logistic regression analysis; [2]The mixed type included 0-Ⅰs + Ⅱc, 0-Ⅱa + Ⅱc, 0-Ⅱc + Ⅱa and 0-Ⅱc + Ⅲ in this series; [3]One or several ulcer plaques/scars at least 5 mm in size.

DISCUSSION

Dysplasia is now replaced by IEN to indicate intraepithelial neoplastic changes with the absence of stromal invasion. It was reported that 0%-23% of mild dysplasia might progress to invasive cancer while the incidence of severe dysplasia is estimated to be 70%-85% in Western studies[1-7,16] and 10% in Japanese reports[8]. Most of these studies focusing on long-term follow-up results had a limited number of patients enrolled. Furthermore, the initial patient status is usually decided by endoscopy, which may fall into the pit of underestimation caused by inevitable biopsy bias.

China is an East Asian country with a high risk of gastric cancer[9]. The incidence of age-adjusted gastric cancer in males and females was 46.5 and 21.0 per 100 000 in Shanghai, China in 1996[17]. The number decreased to 27.4 and 14.0 per 100 000 in 2002 and 2004, still significantly higher than that in most Western countries and Japan[18]. The number of gastric cancer patients per year in the authors" institution, however, has increased significantly in recent years with a total of 2355 cases of surgically proved gastric cancer from January 2001 to March 2008[19]. In addition, intestinal-type gastric carcinoma, a consequence of HGIEN if left untreated[20], is found more common in high risk areas than in low risk areas of gastric cancer. In this study, all patients received curative resection at a mean interval of 19 d between the diagnosis of HGIEN and resection, and invasive carcinoma was found in up to 77.8% lesions. It was reported, however, that the time frame between initial diagnosis of severe dysplasia

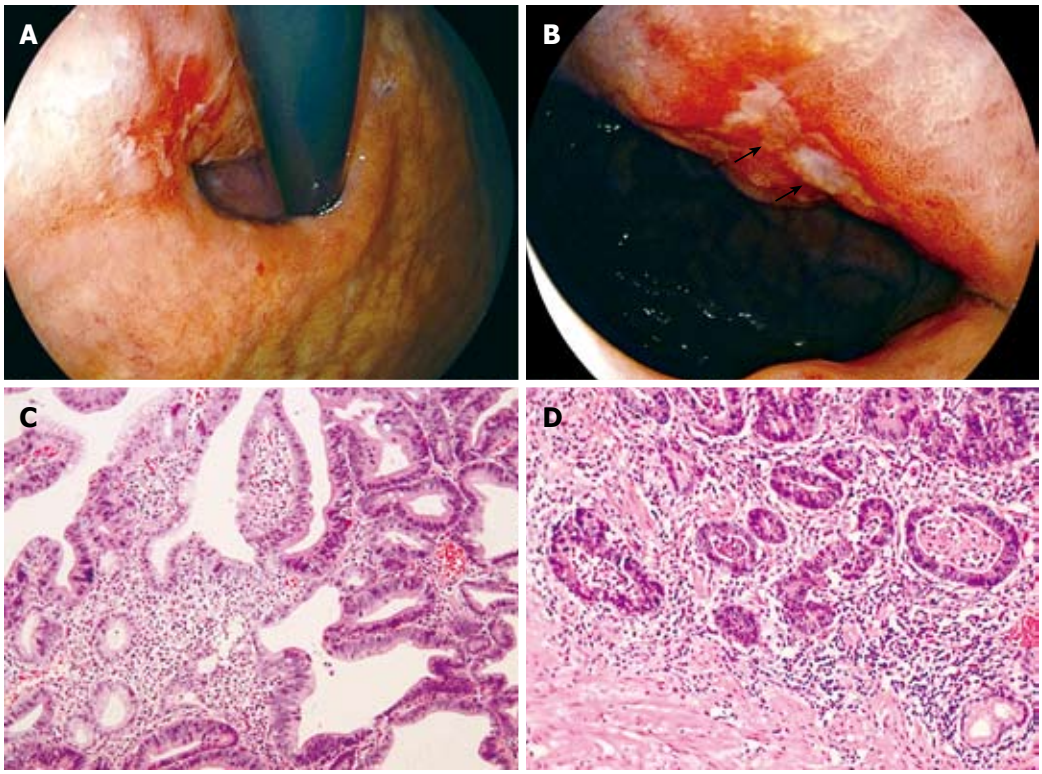

Figure 2 Presence of ulcer plaque is associated with gastric cancer. A: Retroflex view of a flat depressed lesion in the lesser curvature of cardia. The lesion is presented with a reddish area approximately 1.4 cm in size, scattered with irregular ulcer plaque; B: Forward view of the same lesion. The ulcer plaque can be clearly observed with a size larger than 5 mm (arrows); C: Low-power view of biopsy specimen shows irregular tubules with increased branching and architectural distortion. Prominent cellular atypia can be noted, indicating high grade intraepithelial neoplasia (HE staining, × 100); D: The entire lesion was removed by surgery which showed tumor invasion into lamina propria and partly the muscularis mucosae (HE staining, × 100). The final diagnosis was a type 0-Ⅱc well-differentiated adenocarcinoma with muscularis mucosae invasion, T1 N0 M0.

and identification of gastric cancer, was between four and twenty-three months on average[2,4-9,21]. It implys that most gastric lesions diagnosed as HGIEN (severe dysplasia) are underestimated and a lot biopsy-proven "HGIEN" lesions actually are gastric cancer in the meantime. The process of HGIEN advancing to gastric cancer may not be what meets the eye through repeated biopsy.

Although the diagnosis of HGIEN was carefully considered in the study to rule out possible invasion in biopsy samples, it was almost inevitable to get rid of biopsy bias. Multiple causes lead to biased biopsy sampling, including missed target and inadequate tissue amount. Active inflammation of the tissue sample may conceal neoplastic architectural distortion and lead to false negative results. Short-course (2 wk) proton pump inhibitor treatment is suggested for such condition and re-biopsy should be performed. A combination of well-differentiated cancer tissue and absent muscularis mucosa is a typical case in a biopsy sample leading to a diagnosis of HGIEN. The reason why there were not so many well-differentiated adenocarcinomas in this series is probably due to the fact that gastric cancer tends to be less differentiated as it penetrates into deeper layer[22]. It was reported that as high as 11% of biopsy-proven differentiated early gastric cancer turns out to be undifferentiated at surgery[23]. Cryptal dysplasia is another case in which atypia originates from deeper portion of the mucosa and penetrates downward; the epithelium is sometimes spared with little trace of IEN[21]. In this series, only 3.6% of the lesions were proved to be undifferentiated gastric cancer, indicating that this histological type is unlikely to present in biopsy-proven HGIEN.

Macroscopically, superficial changes or markedly ulcerated appearance can be found in a lesion diagnosed as HGIEN by biopsy. However, all "true HGIEN" lesions in this series were less than 20 mm in size while 87.5% of which were less than 15 mm in size. To draw a definite line between HGIEN and gastric cancer is almost unworkable under endoscope, yet a marked lesion suggestive of Borrmann-type gastric cancer always turns out to be invasive cancer[3]. We hereby propose a macroscopic criterion for true HGIEN which can better differentiate true HGIEN from superficial gastric cancer. The criterion involves endoscopic and biopsy parameters easy to be measured in daily practices with conventional endoscope and without the aid of complicated techniques. The relatively low predictive value (70%) for true HGIEN is primarily attributed to the overlapping macroscopic feature of HGIEN and early gastric cancer, both of which can be small and inconspicuous, yet the former seems much unlikely to be a large one. Since HGIEN is a precancerous change without stromal invasion or lymph node metastasis, endoscopic *en-bloc* resection, both preserving gastric

function and improving quality of life of patients, is considered sufficient and curative.

The authors hold that an ulcerated lesion with biopsy-proved HGIEN should always turn out to be invasive cancer, which is supported by the theory of ulcerated early gastric cancer progression proposed by Kyoichi[24], in which the Hauser's cancer is considered a sporadic case. In this series, although the comparison of ulcerated type between the groups did not reach a level of statistical significance, all the patients (8/8) were proved to have invasive cancer. The lack of significance could be attributed to inadequate population size. Same explanation also goes to the issue of mucosal ruga changes, which has been recognized as signs indicating submucosal tumor invasion[24,25]. Experienced pathologists are warranted in making the proper diagnosis of HGIEN without confusion of regenerative changes associated with erosion or healing ulcer or cases suggestive of gastric cancer with inadequate tissue.

In conclusion, a superficial gastric lesion diagnosed as HGIEN by biopsy is most likely to be an early gastric cancer at the time of diagnosis. Lesions greater than 1.5 cm in size and presence of 5-odd mm ulcer plaque or ulcer scar are independently associated with invasive cancer, especially differentiated gastric carcinoma in such a setting. Combining endoscopic characteristics with biopsy results could greatly enhance the diagnostic accuracy of superficial gastric neoplastic lesions, thus reducing the risk of unnecessary gastrectomy, simplifying patient work-up and improving the quality of life of patients. Further investigations are, however, necessary to validate these contentions and to clarify the impact of repeated biopsy on the diagnosis of true HGIEN lesions.

COMMENTS

Background

Intraepithelial neoplasia has been proposed by the WHO to replace dysplasia in gastrointestinal epithelial neoplastic changes. However, differential diagnosis between high grade intraepithelial neoplasia and invasive cancer is still confusing when the diagnosis is based on biopsy pathology.

Research frontiers

Both intraepithelial neoplasia and early gastric cancer can be presented as superficial lesions. Conventional endoscopy provides direct observation of the lesion, yet the appearance of intraepithelial neoplasia and early gastric cancer resembles each other. It is unhelpful to macroscopically differentiate intraepithelial neoplasia from early cancer. Biopsy pathology may also fail to differentiate intraepithelial neoplasia from early cancer. This study focused on the macroscopic features of gastric lesions with a biopsy diagnosis of high grade intraepithelial neoplasia, suggesting that certain macroscopic features can help for better differentiation.

Innovations and breakthroughs

Conventional endoscopy observation has not been considered to play a role in differential diagnosis between intraepithelial neoplasia and early cancer. Likewise, gastric pit pattern observation either by magnifying endoscopy or by NBI lacks the accuracy to better characterize the lesion. Our study suggested that under typical conditions conventional endoscopy would be capable of differentiating between high grade intraepithelial neoplasia and gastric cancer.

Applications

The results of this study enhance the value of routine conventional endoscopy in the diagnosis of clinically confusing conditions. It could simplify the work-up of patients with a biopsy diagnosis of high grade intraepithelial neoplasia and save costs for patients and healthcare system.

Terminology

Intraepithelial neoplasia means to replace dysplasia for precancerous status with cellular and structural atypia. High grade intraepithelial neoplasia denotes increasing architectural distortion with prominent cell atypia without invasion to lamina propria.

Peer review

The authors investigated the macroscopic features of gastric superficial lesions with a biopsy diagnosis of high grade intraepithelial neoplasia and compared endoscopic findings with surgical results. It revealed that a lesion > 1.5 cm in size and the presence of ulcer/scar are associated with invasive cancer. The results are of interest and value for better patient work-up.

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BRIEF ARTICLES

Fast track clinical pathway implications in esophagogastrectomy

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Abstract

AIM: To investigate the feasibility of fast track clinical pathway for esophageal tumor resections.

METHODS: One hundred and fourteen patients with esophageal carcinoma who underwent esophagogastrectomy from January 2006 to October 2007 in our department were studied. Fast track clinical pathway included analgesia control, fluid infusion volume control, early ambulation and enteral nutrition. Nasogastric tube was removed 3 d after operation and chest tube was removed 4 d after operation as a routine, and full liquid diet 5 d after operation.

RESULTS: Among 114 patients (84 men and 30 women), 26 patients underwent fast track surgery, including 17 patients over 65 years old and 9 under 65 (P = 0.014); 18 patients who had preoperative complications could not bear fast track surgery (P < 0.001). No significant differences in tolerance of fast track surgery were attributed to differences in gender, differentiated degree or stage of tumor, pathological type of tumor, or operative incision. The median length of hospital stay was 7 d (5-28 d), 4% patients were readmitted to hospital within 30 d of discharge. Three patients died and postoperative mortality was 2.6%. All 3 patients had no determinacy to fast track surgery approach.

CONCLUSION: The majority of patients with esophageal carcinoma can tolerate fast track surgery. Patients younger than 65 or who have no preoperative diseases have the best results. Median length of hospital stay has been reduced to 7 d.

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Key words: Fast track surgery; Esophageal carcinoma; Esophagogastrectomy

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INTRODUCTION

Recently, a new surgical model, fast track surgery, appears[1,2]. Fast track surgery combines various new techniques and new theories used in pre-operative, peri-operative and post-operative care of patients. The methods include anaesthesia, nutritional monitoring, optimal pain control and surgical techniques[3]. The combination of these approaches lowers the stress response, morbidity as well as mortality rates, thus greatly reducing the time required for full recovery. Fast track surgery has significantly changed many clinical therapeiutic modes.

To date, few existing data can prove whether such concept could also be safely applied to esophageal surgery such as esophageal tumor resection[4]. This study reviews the outcome of a single-center survey regarding the application of the new "fast-track surgery" model and its effects in patients undergoing esophagus tumor resection.

MATERIALS AND METHODS

General data

The records of patients with esophageal carcinoma who

underwent esophagogastrectomy from January 2006 to October 2007 in our department were studied. All the patients received preoperative examinations including gastroscope, barium meal of upper gastrointestine, abdominal ultrasonography, chest CT, pulmonary function and hematological examinations.

Operative indications

The diagnosis of esophageal carcinoma was identified, and no obvious surgical contraindications were detected. There was no evidence of metastasis in intrathoracic lymph nodes, such as the signs of recurrent laryngeal nerve paralysis, diaphragmatic paralysis or Horner's syndrome. There was no supraclavicular and cervical lymphadenectasis. If distant metastasis had been found or resection was impossible temporarily, neoadjuvant chemotherapy or radiotherapy was suggested preoperatively. One hundred and fourteen cases were selected according to the surgical criteria. All these patients received preoperative healthy education: (1) respiratory function exercise to improve pulmonary ventilation function; (2) effective cough and expectoration to promote lung reventilation postoperatively; and (3) diet guide to help patients switch from full liquid diet to semiliquid diet after operation. Preoperative preparations: Patients were fasted for 8 h and supplemented with sugar and salt through intravenous infusion 2 h before operation to increase energy. Anesthesia: Double channel lumen intubation general anesthesia combined with epidural block was used. Anesthetists actively removed sputum, closely controlled the intake and output liquid to maintain the stabilization of effective circulation. A classic radical correction of esophageal carcinoma was performed through opening the chest between ribs and preserving rib bed. Intercostal nerves were removed for assisted analgesic effect. A mechanical anastomosis was performed between the end of the esophagus and the side of the stomach. Jejunal tube was placed at 20-30 cm away from distal Treitz ligament. Both thoracic and celiac lymph nodes were excised referring to the Korst's profile of lymph nodes of esophageal carcinoma[5]. Patients were transferred to anesthesia recovery room after operation. The tracheal intubations were removed when their spontaneous breath, stable circulation, cough reflex, swallowing reflex, and oxygen saturation recovered. But patients with histories of severe diseases such as coronary artery diseases or ventilation dysfunction would be sent to the ICU for tracheal extubation. Enteral feedings combined with parenteral alimentation were used for postoperative patients. Parenteral alimentation was supported with enteral feedings via the jejunostomy tube, primarily with a rate of 20 mL/h in the first 48 h after operation and 65 mL/h 2 d later and then with parenteral alimentation. If swallow showed no leak, full liquid diet was started on the fifth day after operation. Patient controlled analgesia was suggested to be a common practice for 48-72 h after operation. Discharge standard: taking food totally through mouth without intravenous infusion and walking freely in rehabilitation home. The first telephone follow-up was carried out in 72 h after the patients discharged from hospital, with regular visits one week after the operation as the clinical follow-up. Later telephone follow-up was done once every week, until 30 d after operation.

Major complications were defined as any complication excluding transient atrial arrhythmias, urinary retention, or nonoperatively managed air leak. Mortality was defined as any death occurring during the hospital stay or within 30 d postoperatively. Outcome data were analyzed by SPSS 13.0 package using Chi-square test or Fisher exact test. $P < 0.05$ was considered to be significant.

RESULTS

Clinical characteristics of patients with esophageal carcinoma

There were 114 patients, 84 men and 30 women. The ratio of male to female was 2.8: 1. The average age was 58.6 years, ranging from 38 to 85. Lesions were located in distal thoracic esophagus in 60 cases (52.6%), in midthoracic esophagus in 35 cases (31.6%), and in upper thoracic esophagus in 18 cases (15.8%). Twenty-eight of 114 cases had histories of various diseases: 14 with respiratory diseases (12.3%), 9 with cardiocerebrovascular disease (7.9%), 8 with other diseases (7.0%), and 3 with more than two kinds of diseases. Twenty-six patients could not undergo the fast track surgery. Seventeen patients were over 65 years old and nine were under 65 ($P = 0.014$); 18 patients who had a preoperative complication could not bear fast track surgery ($P < 0.001$). No significant differences in tolerance of fast track surgery were attributed to differences in gender, degree of differentiation, stage of tumor, pathological type, or operative incision (Table 1).

Anesthsia and analgesia

Sixty percent of the patients received tracheal extubation immediately at the end of the operation, 30% received tracheal extubation a few hours later in recovery room, and 10% demanded mechanical ventilation. The fluid infusion was controlled between 1.5 L and 2.5 L, between 2.5 L and 3.5 L and above 3.5 L for 71%, 25% and 4% of the total number of patients, respectively. A total of 109 (96%) patients accepted PCA, including vein PCA (44%), epidural PCA analgesia (52%) and 5 patients refused PCA for various reasons. PCA lasted 48-72 h ordinarily (Table 2).

Postoperative daily guideline (Table 3)

Postoperative patients relied mainly on enteral feedings and assisted by parenteral alimentation. During the first 48 h after operation, parenteral alimentation was primarily supported with enteral feedings via the jejunostomy tube with a rate of 20 mL/h 2 d later, jejunostomy tube feedings with a rate of 65 mL/h were given priority with supplement of parenteral alimentation. If swallow showed no leak, full liquid diet was started on postoperative day 5. All the patients were educated to prevent aspiration. Nasogastric tube was removed on postoperative day 3 and chest tube was routinely removed on postoperative

Table 1 Patient characteristics

Characteristics	Patients	Complications	Failed fast track	*P* value
Overall	114	19	26	
Gender				0.80
Male	84	14	20	
Female	30	5	6	
Age (yr)				0.014
⩾ 65	50	11	17	
< 65	64	8	9	
Differentiation				1.00
Low	18	4	4	
Moderate	56	8	13	
High	40	7	9	
Stage of cancer at time of surgery				0.58
Ⅰ	8	0	0	
Ⅱ	16	2	4	
Ⅲ	78	14	18	
Ⅳ	12	3	4	
Preoperative complication				< 0.001
Yes	28	12	18	
No	86	7	8	
Pathology				1.00
Squamous cell carcinoma	87	15	20	
Adenocarcinoma	18	3	4	
Other	9	1	2	
Operative incision				0.73
One	60	9	12	
Two	36	6	9	
Three	18	4	5	
Tolerated fast track surgery				0.001
Yes	88	5		
No	26	14		

Table 2 Anesthesia and pain management

Variables	Data
Extubation	114
Immediate	68
Operation day	34
Later	12
Pain management	109
Epidural PCA	59
Vein PCA	50
Operative transfusion	114
1.5-2.5 L	81
2.5-3.5 L	28
> 3.5 L	5

day 4. Patients were encouraged to walk and cough effectively. Respiratory tract care was strengthened in order to promote lung reventilation postoperatively. The average length of hospital stay was 7 d (5-28 d), and 4% of the patients were readmitted within 30 d of discharge (Figure 1).

Major complications

Postoperative mortality was 2.6% (3 cases). Nevertheless, all 3 patients had no relation to fast track surgery. The causes of death: respiratory failure in two patients and multiple organ failure in one. Nineteen patients had a variety of 34 major complications: 38% with pulmonary complications, 26% with cardiac complications, 15% with anastomotic complications, and 21% with other complications (Table 4).

Table 3 Daily guideline of postoperative care of patients with fast track surgery

Day	Daily guideline of postoperative care
POD 1	Jejunal tube feeding 500 mL (20 mL/h);physical therapy four times per day;chest tube and nasogastric tube draining patency; head of bed put at 45-60 degree; supply albumin
POD 2	Jejunal tube feeding 1000 mL (40 mL/h) ;remove urinary catheter and epidural (Or pod3 remove);encourage patient to ambulate; chest tube and nasogastric tube draining patency; continue physical therapy, promoted to lung recruitment
POD 3	Jejunal tube feeding 1500 mL (65 mL/h); remove nasogastric tube; continue physical therapy
POD 4	Jejunal tube feeding 1500 mL (65 mL/h); X-ray; remove chest tube (drainage < 100 mL)
POD 5	Jejunal tube feeding 1500 mL (65 mL/h); gastrografin swallow; anastomosis showed no leak; advice patient to take a little liquid diet; education on aspiration precaution
POD 6	Increase liquid diet; continue to jejunal tube feeding
POD 7	Remove jejunal tube; full liquid diet

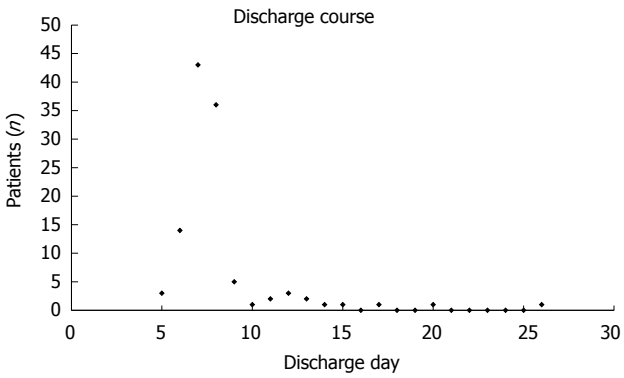

Figure 1 Patients with fast track surgery discharge course.

DISCUSSION

Fast track surgery was suggested by Henrik Kehlet *et al*[1,2] who thought postoperative degression process of body function could be shortened from a few weeks to a few days. One hundred and fourteen patients undergoing fast-track rehabilitation got good therapeutic effect, compared with the traditional surgery of esophageal cancer[6-9] (Table 5) .Fast track surgery were successful in many general surgical patients, especially in colorectal surgery patients[10-12]. In our study, postoperative hospital stay was significantly shortened and cost of hospitalization was reduced without increase of morbidity or mortality. Postoperative hospital stay was declined from traditionally 12-14 d to now 7 d through the use of fast track surgery. Multivariate analysis showed that age (> 65 years) and

Table 4 Postoperative morbidity

Variables	Data
Pulmonary	13
Empyema	2
Atelectasis	2
Pulmonary edema	1
Pulmonary infection	3
Respiratory insufficiency	5
Anastomotic	5
Leak	4
Hemorrhage	1
Cardiac	9
Atrial arrhythmia	4
Supraventricular arrhythmia	3
Ventricular arrhythmia	2
Other	7
Chylothorax	3
Hoarseness	2
Gastric perforation	1
Ileus	1
Mortality	3
Respiratory failure	2
Multiple organs failure	1

preoperative complications were the main factors that determined whether patients could bear fast track surgery clinical pathway.

In brief, fast track surgery uses multiple modes to control pathophysiological process of perioperative patients. It can improve the prognosis and promote their recovery. How to attenuate the stimulation is the basis of reduction of stress response. Basically, the approaches include three aspects: (1) make good physical and mental preparation preoperatively; (2) minimize therapeutic stress; and block nerve conduction to stress signal[13]. In a wide view of these measures, there are not many higher demands of surgical skills. Fast track surgery approach mainly optimizes perioperative management on the basis of various effective methods confirmed by evidence-based medicine. As a result, fast track surgery reduces common complications, relieves patients' pain and accelerates patients' recovery.

Intravenous nutrition, gastrointestinal decompression and chest tube are all necessary for traditional esophagogastrectomy. Postoperative pain is common. We used fast track surgery to optimize traditional measures. It is known that gastrointestinal decompression and delayed eating are conventional means. However, nasogastric tube made 88% patients discomfort to a moderate to severe degree, and 70% patients with even more severe feelings[14,15]. It is obvious that functional restoration of gastrointestine was delayed. Some studies found that cancellation of gastrointestinal decompression might be a vital step for faster functional restoration of gastrointestine and decrease of hospital stay. A recent meta-analysis showed that gastrointestinal decompression had not reduced the complications of operation on digestive tract[16]. Nevertheless, our research indicated that function of gastrointestine was in the stage of overall recovery during 48 after operation, therefore, gastrointestinal decompression would be

Table 5 Published esophagogastrectomy outcomes (recently 5 years)

Source	Patient	Postoperative morbidity (%)	Postoperative morbidity (%)	Mean length of hospital stay (d)
Goan *et al*[6] (1994-2005)	216	49.0	9.7	None
Low *et al*[7] (1991-2006)	340	45.0	0.3	11.5
Ancona *et al*[8] (1992-2002)	522	16.3	None	14.0
Atkins *et al*[9] (1996-2002)	379	64.0	5.8	15.0

helpful. In our opinion, gastrointestinal decompression is essential in the early postoperative stage, and the discomfort can be relieved by drugs.

Pulmonary complications are not only the most common but also the main death cause after operation[17]. So how to effectively prevent pulmonary complications becomes the focus of fast track surgery. Satisfying health education could relieve patients' anxiety and fear, and partially reduce postoperative pain. Patients could benefit from good partner treatment and sufficient cardiorespiratory function exercise preoperatively. Full communication with anesthetist perioperatively is very helpful for anesthetist to grasp patient's condition. On the premise of ensuring effective circulation, anesthetist controlled the fluid infusion volume, actively sucked sputum, and gave early tracheal extubation to promote lung reventilation postoperatively. Recently, the evidence shows that the reduction of fluid volume in the operation would help reduce postoperative complications and shorten the hospital stay[18,19]. After operation, anesthetist performed a various kinds of patient control analgesia. Postoperative analgesia is not only the demanding of humanism but also the vital component of fast track surgery. It is very helpful for patients to ambulate earlier so that muscles will not be hypertrophied because of long-term bed-ridden. It is also very helpful for patients to cough and expectorate effectively to recruit their lungs. This could reduce thrombogenesis and pulmonary infection. Epidural analgesia has advantages in the early recovery of pulmonary function and early physical therapy. And effective cough for the effect of epidural analgesia was regional and weaker compared with vein analgesia. But the manipulation of epidural analgesia is complex. It is hard to control anesthesia plane and its effect is instable. We have found that the cooperation of surgeons, anesthestists, and dietitian is very important because they can work together with multiple means to effectively decrease the complications of lung and heart.

Early enteral nutrition not only gurantee the structure and function of intestinal mucosa cells, maintain intestinal mechanical, chemical and biological barrier, but also increase portal circulation, activate intestinal digestive secretary system and accelerate organ recovery. Enteral nutrition has also been an important measure for fast-track. Now perioperative nutritional support

contains the full regulation of pathophysiological process as well as simple nutritional regulation. In the past decade, advantage of diversion from parenteral nutrition to enteral nutrition has been generally accepted. Enteral nutrition is more suitable for physiological demanding with less complications and lower price. Enteral alimentation should be small in amounts and short in time. Parentral nutrition is the major supplement and the whole enteral nutrition should be given 48 h later. Immunological enteral nutrition could be used for some indicated patients. A recent meta-analysis demonstrated that immunological enteral nutrition could reduce postoperative infection complications[20].

Enteroparalysis and anastomotic leakage have been two major puzzles for early eating after operation. Enteroparalysis was considered to be inevitable in the past. There are a few factors related to enteroparalysis which could be ameliorated by intestinal rest and gastrointestinal decompression. However, some studies indicated that intestinal rest and nasogastric tube decompression were not indispensable[21,22]. That fast track encourages patients to have a diet early did not increase discomfort but promoted the intestinal recovery. Postoperative anastomotic leakage was a dangerous complication in the past. Late diet was thought to prevent leakage. However, with the development of evidence-based medicine, there has been no proof that late diet can reduce this kind of complication. Recent research showed that severe complications were mainly related to the condition of patients before operation and surgical techniques[23]. In our study, 4 cases had leakage in neck and 1 case had leakage in the chest. But no evidence indicated leakage related to early diet.

Age and preoperative disorders are also the key factors influencing whether patients can tolerate fast track surgery. Organic functions of patients over 65 years of age are in the degenerating stage. They had intense stress caused by the operation and so their recovery was relatively slow. Fast track surgery would increase their complications and had a disadvantage in early restoration. Patients with preoperative disorders especially heart and lung diseases might fail in the fast-tracking protocol. So it is suggested that the primary diseases should be ameliorated actively and cardiac-pulmonary functions should improve before operation. If organ disorders and nutrition status achieve good improvement, patients can accept fast track surgery mode.

Our research demonstrated that the majority of patients with esophageal carcinoma can tolerate fast track surgery clinical pathway. Patients less than 65 years or without preoperative diseases have the best effect. Average length of hospital stay has been reduced to 7 d. The major goal of fast track surgery is to control pathophysiological process and improve patients' prognosis, not only for early discharge, but also for early recovery. Fast track surgery is not only the responsibility of surgeons, it also needs a team that could cooperate effectively. This involves surgeons, anesthestists, dietitians, and nurses[24]. Fast track surgery clinical pathway increases patients' satisfaction and reduce costs. It can also use medical resources in the most efficient way. As a result, fast track surgery will be the tendency of surgery development.

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COMMENTS

Background

A new surgical model, fast track surgery, has appeared recently. Many clinical therapeutic modes have been significantly changed due to fast track surgery. However, the mechanism of this treatment in esophageal tumor resection is still unclear.

Research frontiers

To date, few existing data can prove whether such concept in fast track surgery could be safely applied to esophageal surgery such as esophageal tumor resection. This study reviews the outcome of a single-center survey regarding the application of the new "fast-track surgery" clinical pathway and its effects in patients undergoing esophagal tumor resections.

Innovation and breakthroughs

Patients were preoperatively fasted for 8 h only and supplemented with sugar and salt by intravenous infusion 2 h before operation to increase energy. Postoperative patients relied mainly on enteral feedings and were assisted by parenteral alimentation. Nasogastric tube was removed on postoperative day 3 and chest tube was removed on postoperative day 4.

Applications

The majority of patients with esophageal carcinoma can tolerate fast track surgery clinical pathway. Patients less than 65 years of age or without preoperative diseases have the best effect in the treatment.

Terminology

FTS stands for fast-track surgery, a combination of various new techniques which reduce the stress response as well as morbidity and mortality rates, thus greatly shortening the time required for full recovery.

Peer review

Several articles have evaluated fast-tracking pathways. The primary objective of this study was to determine if fast-tracking pathway can be applied with satisfaction without increasing morbidity or mortality as compared to other reports. The initial observation is interesting.

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CASE REPORT

Postpartum spontaneous colonic perforation due to antiphospholipid syndrome

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Abstract

The antiphospholipid syndrome (APS) is a multi-systemic disease being characterized by the presence of antiphospholipid antibodies that involves both arterial and venous systems resulting in arterial or venous thrombosis, fetal loss, thrombocytopenia, leg ulcers, livedo reticularis, chorea, and migraine. We document a previously unreported case of a 37-year-old female in whom APS was first manifested by infarction and cecal perforation following cesarean section. At laparotomy the underlying cause of colonic perforation was not clear and after resection of the affected bowel an ileo-colostomy was performed. The diagnosis of APS was established during post-operative hospital stay and the patient was commenced on warfarin. Eventually, she made a full recovery and had her stoma reversed after 4 mo. Pregnancy poses an increased risk of complications in women with APS and requires a more aggressive approach to the obstetric care. This should include full anticoagulation in the puerperium and frequent doppler ultrasound monitoring of uterine and umbilical arteries to detect complications such as pre-eclampsia and placental insufficiency.

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Key words: Antiphospholipid syndrome; Intestinal ischemia; Acute abdomen; Pregnancy

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INTRODUCTION

Antiphospholipid syndrome (APS) is characterized by the presence of antiphospholipid antibodies and multiple systemic abnormalities including arterial or venous thrombosis, fetal loss, thrombocytopenia, leg ulcers, livedo reticularis, chorea, and migraine[1-5]. Venous thrombosis commonly involves deep venous system of the leg, but the renal vein, pulmonary vein, inferior vena cava, hepatic vein, and portal vein may also be involved[2,6,7]. The most common site of arterial thrombosis is the cerebral circulation, but occlusion of coronary, renal, or retinal arteries has also been reported[6,7]. Only rarely has mesenteric arterial thrombosis been noted[1,6]. We report an unusual case where APS was first manifested by infarction and cecal perforation following cesarean section.

CASE REPORT

A 37-year-old lady was admitted for elective induction of labor, but proceeded to have an emergency cesarean section due to prolonged second stage of labor. During the postpartum period she developed abdominal distention and exhibited signs of peritonitis. Laboratory tests showed a leukocyte count of 21 × 10^9 with 19 × 10^9 neutrophils, hemoglobin of 11.9 g/dL and INR of 1.15. Renal and liver biochemistries were normal. Computer-

Figure 1 CT revealed free gas and fluid in the abdomen.

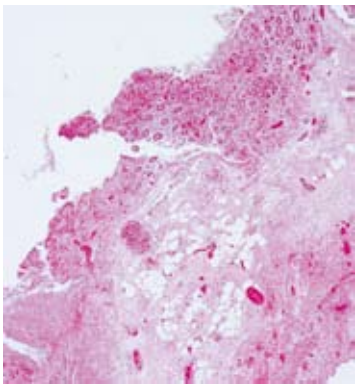

Figure 2 Histopathology showed bowel wall necrosis.

ized tomography (CT) revealed a large amount of free gas and free fluid in the abdomen indicative of bowel perforation (Figure 1).

After initial resuscitation, she underwent emergency laparotomy which revealed generalized fecal peritonitis and multiple perforations of the cecum. Macroscopically the entire right colon looked inflamed. After peritoneal lavage a right hemicolectomy was performed. A double barrelled stoma (ileo-colostomy) was fashioned due to fecal soiling of the peritoneal cavity and unknown etiology of the perforation. Post operative recovery was complicated by intra-abdominal serous collections, necessitating percutaneous drainage, and pleural effusion for which she needed a chest drain insertion.

Histopathology of the resected bowel revealed transmural infarction with the presence of occasional fibrin thrombi in adjacent blood vessels without evidence of vasculitis (Figure 2). Background bowel and the resection margins showed peritonitis, but the wall was viable and did not show any significant abnormalities.

Clotting profile confirmed the presence of lupus anti-coagulant, but it was negative for anticardiolipin antibodies. Magnetic resonance angiography (MRA) revealed occlusion of the inferior mesenteric artery and stenosis of the hepatic artery. Ultrasound scan of the hepatobiliary system was normal. A diagnosis of APS was established and she was subsequently commenced on life-long warfarin (target INR 3-4).

The stoma was closed 4 mo later after a normal contrast enema. The patient made an uneventful recovery and remains well on follow-up.

DISCUSSION

APS is characterized by a state of hypercoagulability potentially posing a risk of thrombosis to all segments of the vascular bed and may result in pregnancy related morbidity. It is associated with the presence of a specific group of autoantibodies called antiphospholipid antibodies (aPL) which are circulating immunoglobulins that cross-react with cell membrane phospholipids. The two main types of aPL are the anticardiolipin antibodies (aCL) and the lupus anticoagulant (LA). These antibodies are found in 2% of the general population and in 30%-40% of systemic lupus erythematosus patients[7,8]. Although patients with syphilis, acquired immunodeficiency syndrome or other connective tissue disorders may have these antibodies, they may not manifest clinical features. About 30% of patients with the LA and 30%-50% with high or medium positive IgM aCL antibodies have clinical features of APS. The exact prevalence remains unknown[9]. The aCL test is positive in about 80% of patients with APS, the LA positive in approximately 20%, and both are present in 60% of the cases[10]. The Sapporo criteria require the positivity on two occasions, at least 6 wk apart of aCL at medium-high titres or LA[11]. However, this was recently revised to 12 wk by international consensus[12].

Anticardiolipin antibodies are strongly associated with thromboembolic phenomenon, thrombocytopenia, and prolonged prothrombin and partial thromboplastin times[8]. The suggested mechanisms for reaction of antibodies with epitopes are anticardiolipin binding to acidic phospholipids on platelet membranes resulting in platelet activation[13,14], anticardiolipin blocking prothrombinase activation, anticardiolipin binding to membrane phospholipids to cause endothelial cell injury, and anticardiolipin alteration of the local endothelial levels of prostacyclin, prekallikrein, antithrombin III, or protein C[15-18]. However, lupus anticoagulants are a stronger risk factor for thrombosis than anticardiolipin antibodies in APS[19]. This was the case in our patient who was anticardiolipin negative, but lupus anticoagulant positive.

A wide range of abdominal manifestations of APS have been reported, with hepatic involvement being the most common, followed by thrombotic events involving different branches of the intestinal vasculature[20]. Sporadic splenic infarction and acute pancreatitis have also been reported. A wide range of hepatic diseases are associated with APS including both thrombotic (Budd-Chiari syndrome and hepatic infarction) and non-thrombotic conditions (cirrhosis, autoimmune hepatitis and portal hypertension). Intestinal infarction, due to thrombosis of mesenteric vessels, has been relatively less frequently reported in patients with APS[21].

Cases of mesenteric venous thromboses presenting with intestinal ischemia in patients with APS have been

reported in the literature[22]. The presentation may be acute, often preceded by intestinal angina, or rarely results in intestinal bleeding and ulceration[20]. The occurrence of celiac artery and/or superior mesenteric artery stenosis has also been documented[23].

Computerized tomography (CT) is considered to be first-line investigation in APS patients presenting with abdominal symptoms. CT features of mesenteric ischemia have been reported to show thickening of both small and large bowel walls with prominence of the supplying mesenteric vessels[24]. Mesenteric MRA (magnetic resonance angiogram) in cases of suspected intestinal ischemia may confirm the diagnosis if the patients' condition allows. In our case, the patient underwent an emergency laparotomy and spontaneous perforation of the cecum was found. The underlying cause, however, was not clear at the time of surgery and the diagnosis of APS was made during post-operative hospital stay.

Venous or arterial intestinal thromboses and infarctions may be prominent features of APS. The presentation may be non-specific and a high index of suspicion is needed for any signs of abdominal involvement in these patients. Pregnancy poses increased risks of complications (e.g. hypertension/pre-eclampsia, prematurity or thrombosis) and requires a more aggressive approach to the obstetric care. This should include full anticoagulation in the puerperium and frequent ultrasound monitoring with uterine and umbilical artery Doppler blood flow analyses to detect complications such as pre-eclampsia and placental insufficiency.

It is recommended that all women with APS should maintain antithrombotic treatment throughout their entire pregnancy and during the postpartum period. The treatment of choice is combined low-dose aspirin and full antithrombotic doses of low molecular weight heparin. In high risk groups, such as history of previous thrombotic events, warfarin may be used during pregnancy, but only after organogenesis (6th-12th wk) because of high risks of fetal malformations. Despite the significant risks associated with pregnancy in patients with APS, with the correct management the likelihood of a live birth is around 75%-80%[25].

To the best of our knowledge, this is the first report in the English literature of spontaneous colonic perforation due to APS following cesarean section. Our patient was previously fit and healthy and received the standard prophylaxis for thromboembolism with low molecular weight heparin. The underlying APS in combination with pregnancy and surgery, both of which are risk factors for thromboembolism, caused mesenteric thrombosis and cecal infarction.

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LETTERS TO THE EDITOR

nm23H1 expression and its role in the evolution of non-gastrointestinal malignancies

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Abstract

The role of *nm23H1* genetic instability is not limited to gastrointestinal malignancies. A similar close relationship exists between *nm23H1* genetic instability and other non gastrointestinal systemic malignancies. For instance, in oral malignant melanomas with lymphoid metastasis, the *nm23H1* expression is significantly lower in contrast to tumors with no lymphoid metastasis. Similarly, increased metastasis is seen in non small cell lung cancers following down regulation of *nm23H1* in conjunction with KAI-1 down regulation. There is an inverse relationship between tumor stage and metastasis and *nm23H1* expression in individuals with prostate carcinomas and a similar relationship exists between microsatellite instability of the *nm23H1* gene and ovarian carcinogenesis. For instance, nearly 70.5% of stage Ⅰ-Ⅱ ovarian tumors express *nm23H1* in sharp contrast to only 25% of stage Ⅲ-Ⅳ ovarian tumors. As is clearly evident, *nm23H1* has a major role in gastrointestinal and non-gastrointestinal carcinogenesis. The coming few years will hopefully see the development of new strategies by virtue of which we can alter *nm23H1* expression and thus decrease the risk of metastasis in malignant tumors.

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Key words: *nm23H1*; Non small cell lung cancers; Prostate carcinomas; Nasopharyngeal carcinomas

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Kapoor S. *nm23H1* expression and its role in the evolution of non-gastrointestinal malignancies. *World J Gastroenterol* 2009; 15(4): 506-507 Available from: URL: http://www.wjgnet.com/1007-9327/15/506.asp DOI: http://dx.doi.org/10.3748/wjg.15.506

TO THE EDITOR

The recent article by Yang *et al*[1] about the relationship between gastrointestinal malignancies and *nm23H1* genetic instability is highly interesting. Interestingly, the role of *nm23H1* genetic instability is not limited to gastrointestinal malignancies. In fact, a similar close and intricate relationship exists between *nm23H1* genetic instability and other non gastrointestinal systemic malignancies.

For instance, Korabiowska *et al*[2] have shown that the *nm23H1* expression is significantly lower in oral malignant melanomas with lymphoid metastasis than in tumors with no lymphoid metastasis. Similarly, increased metastasis is seen in non small cell lung cancers following down regulation of *nm23H1* in conjunction with KAI-1 down regulation[3]. Similarly, there is an inverse relationship between tumor stage and metastasis and *nm23H1* expression in individuals with prostate carcinomas[4]. A similar relationship exists between microsatellite instability of the *nm23H1* gene and ovarian carcinogenesis. For instance, nearly 70.5% of stage Ⅰ-Ⅱ ovarian tumors express *nm23H1* in sharp contrast to only 25% of stage Ⅲ-Ⅳ ovarian tumors[5].

Similarly, *nm23H1* expression may be used to determine response to treatment. For instance, following radiotherapy, the five-year survival rate in patients with nasopharyngeal carcinomas and high expression of *nm23H1* is 53.2% in comparison to only 22.7% in individuals with low expression of *nm23H1*[6]. In fact, transfer of *nm23H1 via* adeno viruses is rapidly emerging as a potential therapeutic tool to prevent metastasis. For instance, this method has been shown to decrease metastasis in implantation models of ovarian cancer[7].

As is clearly evident, *nm23H1* plays a major role in gastrointestinal and non-gastrointestinal carcinogenesis. The coming few years will hopefully see the development of new strategies by virtue of which we can alter *nm23H1* expression and thus decrease the risk of metastasis in malignant tumors.

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Meetings

Events Calendar 2009

January 12-15, 2009
Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

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

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

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

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

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

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

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

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

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

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

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

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

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

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

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

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

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

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

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

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

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

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
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Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open-access (OA) peer-reviewed journal supported by an editorial board consisting of 1212 experts in gastroenterology and hepatology from 60 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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

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

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EDITORIAL

Dysregulation of apoptosis in hepatocellular carcinoma cells

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Abstract

Hepatocellular carcinoma (HCC) is a major health problem, being the sixth most common cancer world-wide. Dysregulation of the balance between proliferation and cell death represents a pro-tumorigenic principle in human hepatocarcinogenesis. This review updates the recent relevant contributions reporting molecular alterations for HCC that induce an imbalance in the regulation of apoptosis. Alterations in the expression and/or activation of p53 are frequent in HCC cells, which confer on them resistance to chemotherapeutic drugs. Many HCCs are also insensitive to apoptosis induced either by death receptor ligands, such as FasL or TRAIL, or by transforming growth factor-beta (TGF-β). Although the expression of some pro-apoptotic genes is decreased, the balance between death and survival is dysregulated in HCC mainly due to overactivation of anti-apoptotic pathways. Indeed, some molecules involved in counteracting apoptosis, such as Bcl-X_L, Mcl-1, c-IAP1, XIAP or survivin are over-expressed in HCC cells. Furthermore, some growth factors that mediate cell survival are up-regulated in HCC, as well as the molecules involved in the machinery responsible for cleavage of their pro-forms to an active peptide. The expression and/or activation of the JAK/STAT, PI3K/AKT and RAS/ERKs pathways are enhanced in many HCC cells, conferring on them resistance to apoptotic stimuli. Finally, recent evidence indicates that inflammatory processes, as well as the epithelial-mesenchymal transitions that occur in HCC cells to facilitate their dissemination, are related to cell survival. Therefore, therapeutic strategies to selectively inhibit anti-apoptotic signals in liver tumor cells have the potential to provide powerful tools to treat HCC.

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INTRODUCTION

Apoptosis represents a physiological way to eliminate excess cells during both liver development and regeneration[1]. Indeed, insufficient apoptosis has been associated with development and progression of tumors of the liver and the biliary tree[1,2]. Hepatocellular carcinoma (HCC) is a major health problem, being the sixth most common cancer world-wide[3]. It is a heterogeneous tumor commonly associated with chronic liver diseases which frequently culminate in cirrhosis, such as alcoholic cirrhosis and chronic hepatitis B and C infections. During recent years, major advancements in the knowledge of this complex disease have been reported[3]. This review is an effort to update the recent relevant contributions reporting molecular alterations for HCC that induce an imbalance in the regulation of apoptosis.

THE P53 PATHWAY

Among the most common alterations observed in HCC are mutations in the p53 tumor suppressor gene (*TP53*)[4]. Different chemotherapeutic agents require p53 to induce apoptosis. Indeed, tumors with a disruption in the p53 pathway are generally resistant to chemotherapy. The presence of specific mutational hotspots in *TP53* in different types of human cancer implicates environmental carcinogens and endogenous processes. In this sense, somatic mutations at the third base in codon 249 of *TP53* in HCC have been related to exposure to aflatoxin B1 (AFB1), in association with HBV infection[4]. Chronic infection with HBV and HCV viruses and exposure to oxidative stress, including hemochromatosis or inflammation, induce damage in the DNA and mutations in cancer-related genes, including *TP53*. Thus, it would

seem plausible that p53 mutation might operate in either HCC initiation or progression, depending on the context. However, adenoviral delivery of p53 recombinant DNA into mice models bearing hepatocellular carcinomas did not apparently suppress tumor growth[5]. De-Pinho *et al* in a recent work[6] have helped to clarify this point. They have demonstrated that the effect of p53 loss in hepatocellular carcinoma that is associated with chronic liver disease is dependent on cellular context, in particular intact or dysfunctional telomeres, and they have hypothesized that a decreased p53 function might contribute to hepatocyte survival in the presence of telomere-induced chromosomal instability.

THE TGF-β PATHWAY

The transforming growth factor-beta (TGF-β) family of cytokines plays a physiological role during embryonic development and its misregulation can result in tumorigenesis[7]. TGF-β-1 is an important regulatory suppressor factor in hepatocytes, inhibiting proliferation[8] and inducing cell death[9]. Paradoxically, TGF-β may also modulate other pro-tumorigenic processes, such as cell invasion, immune regulation or microenvironment modification[7]. Blocking TGF-β up-regulates E-cadherin and reduces migration and invasion of hepatocellular carcinoma cells[10]. Furthermore, liver tumors expressing late TGF-β-responsive genes (anti-apoptotic and metastatic) display a higher invasive phenotype and increased tumor recurrence when compared to those that show an early TGF-β signature (suppressor genes)[11]. Indeed, the escape from the antiproliferative and pro-apoptotic actions of TGF-β might be a prerequisite for hepatocarcinoma progression[12].

Disruption of the TGF-β pathway occurs in HCC[13] and might cause dysregulation of apoptosis. In favour of this hypothesis, recent studies have demonstrated that overexpression of SMAD3 reduces susceptibility to develop hepatocarcinoma, by sensitizing hepatocytes to apoptosis through down-regulation of Bcl-2[12]. However, perturbations at receptor or SMAD levels do not appear to be as frequent as they are in colon or pancreatic cancer[13] and expression of TGF-β is up-regulated in a great percentage of HCC patients[11,13]. Thus, other possible ways to disrupt TGF-β signalling might exist and they remain to be explored. Interestingly, Mishra *et al* have recently demonstrated that HCC might arise from loss of TGF-β signalling adaptor protein embryonic liver foldrin (ELF), a crucial Smad3/4 adaptor[14,15]. HCC cells might also overexpress a specific set of microRNAs (miRNAs) that would allow the escape from TGF-β-induced apoptosis[16,17]. Furthermore, recent results have indicated that TGF-β might play a dual role in controlling apoptosis in hepatocytes and hepatoma cells. On one hand, it induces cell death, but on the other it could activate anti-apoptotic signals, the epidermal growth factor receptor (EGFR) being required for this effect[18-20]. Indeed, EGF is an important survival signal for TGF-β-induced apoptosis in hepatocytes[21]. The enzyme phosphatidylinositol 3-kinase (PI3K) mediates the effect of EGF on TGF-β-induced death by acting upstream from the mitochondrial changes, probably counteracting TGF-β-induced oxidative stress[22]. The autocrine loop of EGFR activated by TGF-β in hepatoma cells would require a high activity of TACE/ADAM17[20], the metalloprotease responsible for shedding of the pro-tumor necrosis factor (proTNF-α) that it is also necessary for shedding of the EGF family of growth factors[23]. Although the possible role of an increased expression of TACE/ADAM17 in the development of human hepatocellular carcinoma (HCC) has been barely studied, a recent report indicates that the quantities of ADAM17 mRNA vary among different pathological types of HCC, but are significantly higher in poorly differentiated HCC than in well or moderately differentiated HCC[24]. Overexpression of TACE/ADAM17 might confer an advantage on HCC cells by impairing TGF-β-induced apoptosis through transactivation of the EGFR. Concluding, HCC cells might impair the suppressor arm in TGF-β-signalling, with enhancement of the response to this factor in terms of tumor progression and invasion (Figure 1).

THE DEATH RECEPTOR PATHWAYS

HCCs show resistance to apoptosis mediated by several death receptors. The majority of the HCCs show one or more alterations in the Fas pathway molecules, which inhibit Fas-mediated apoptosis[25]. The status of Fas and Fas ligand (FasL) expression can predict HCC recurrence[26]. Loss of response to Fas in HCC cells may be produced either by down-regulation of *Fas* expression[25,27], concomitant with decreased expression of downstream molecules, such as FADD or FLICE[27], or by up-regulation or over-activation of molecules that counteract its pro-apoptotic effect, including nuclear factor-kappaB (NF-κB), Bcl-2 or Bcl-X_L[28-30]. The cellular FLICE/caspase-8-inhibitory protein (cFLIP), an intracellular inhibitor of caspase-8 activation, is constitutively expressed in human HCC cell lines and displays higher levels in HCC tissues than in nontumor liver tissues[31]. It has also been described that HCC tissues show overexpression of BRE, an antiapoptotic protein that binds to the cytoplasmic domains of tumour necrosis factor (TNF) receptor-1 and Fas, attenuating death-receptor initiated apoptosis[32]. Furthermore, it has been suggested that extracellular factors might counteract Fas-induced apoptosis in HCC cells. Indeed, hepatocyte growth factor (HGF), through activation of the PI3K/AKT pathway, suppresses Fas-mediated cell death in human HCC cell lines, by inhibiting Fas-death-inducing signalling complex (DISC) formation, especially FADD and caspase 8 interaction[33] (Figure 2).

TNF-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in various transformed cell lines but not in almost normal tissues[34]. HCC cells constitutively express TRAIL mRNA and protein, but there are contradictory and confusing data about the expression of the different TRAIL receptors in HCC cells and tissues[35-37]. Certain evidence indicates that most HCC cells are insensitive towards TRAIL-mediated apoptosis, suggesting that the presence of mediators can inhibit the

TRAIL cell-death-inducing pathway in HCC[36,37]. It has been reported that hepatitis B virus core protein inhibits TRAIL-induced apoptosis by blocking the expression of the TRAIL receptor 2 (TRAIL-R2/DR5)[38]. Overactivation of NF-κB and Bcl-X_L in HCC cells might also restrain the TRAIL-mediated apoptosis[39]. After an initial debate about the potential liver toxicity of TRAIL in freshly isolated human hepatocytes[37], there is a recent interest in the development of new therapeutic approaches that can sensitize HCC cells to TRAIL-induced apoptosis. Indeed, it has been proposed that TRAIL, in combination with chemotherapeutic agents, may have potential in the treatment of HCC[40]. Of clinical relevance, proteasome inhibitors and histone deacetylase (HDAC) inhibitors might sensitize HCC cells but not primary human hepatocytes for TRAIL-induced apoptosis[41,42].

ALTERATIONS IN THE EXPRESSION OR FUNCTION OF APOPTOSIS REGULATORY PROTEINS

It is worthy of note that many of the genetic alterations observed in HCC lead to an imbalance in the pro- and anti-apoptotic members of the Bcl-2 family[43]. Bcl-X_L is overexpressed in a great percentage of HCCs[44], and so is Mcl-1[45]. In contrast, pro-apoptotic members of the family, such as Bax or Bcl-X_S are down-regulated in HCC with dysfunction in the p53 pathway[46]. Furthermore, recent results have indicated that some pro-apoptotic members of the BH-3-only family, such as Bid, show decreased expression in HCC related to hepatitis B or C infection[47].

Recent investigations have revealed that nearly 90% of clinical tumors from advanced HCC patients express high levels of X-linked inhibitor-of-apoptosis protein (XIAP), a well known inhibitor of caspases. Studies in established HCC cell lines with different metastatic capabilities indicated a correlation of metastasis with resistance to apoptosis and increased expression of XIAP[48]. Interestingly, it had previously been suggested that XIAP might also function as a cofactor in TGF-β signalling[49]. Thus, overexpression of XIAP might confer resistance to the apoptotic effects of TGF-β, allowing HCC cells to respond to this cytokine in terms of migration and invasion. Genome-wide analyses of tumors in a mouse model of liver cancer and in HCC tissue have recently revealed a recurrent amplification in a region of human chromosome 11q22, delineating *cIAP1*, the known inhibitor of apoptosis, as one of the candidate oncogenes in the amplicon[50]. Survivin, another member of the family of inhibitor of apoptosis proteins, is also overexpressed in HCC cell lines and tissues[51,52] and it has been suggested that it might play a pivotal role in metastasis[53]. Survivin might play an important role in progression of HCC not only by inhibiting apoptosis[54], but also by promoting cell proliferation[51] and may be positively correlated with high risk of disease recurrence and poor prognosis[55]. Concluding, HCC cells show an imbalance in the expression of pro- and anti-apoptotic proteins, which favours cell survival (Figure 2).

OVERACTIVATION OF SURVIVAL SIGNALS IN HCC CELLS

Some autocrine signal activators, such as EGF receptor (EGFR) ligands, might protect liver tumor cells from apoptosis induced by stress, physiological factors or pro-apoptotic drugs[56]. Dysregulation of growth factor signalling, including EGF and IGF-1 pathways, has been well established in human HCCs[57,58]. Viral hepatitis infections might contribute to the enhancement of the expression of EGFR ligands[59]. The tyrosine kinase $p60^{c\text{-}src}$ is also overactivated in hepatoma cells[56,60] that protect themselves from death stimuli[61], and it accounts in a large part for the desensitization of liver tumor cells to TRAIL and CD95. Interestingly, blockade of EGFR or c-Src in primary hepatocytes only marginally increases cell death[56,61], which indicates that both tyrosine kinases are critical effectors that specifically protect liver cancer cells from death stimuli, providing a weak point in cancer cells for a potential therapeutic approach.

Signal transducer and activator of transcription (STAT) proteins become activated by tyrosine kinases in response to cytokines and growth factors. It has been reported that suppressor of cytokine signalling (SOCS)-1 and (SOCS)-3, negative regulators of the JAK2-STAT signalling pathway, are silenced by methylation in human hepatoma cell lines and HCC tissues, which leads to constitutive activation of STAT3 in these cells[62,63]. Deletion of the (*SOCS)-3* gene in hepatocytes promotes the activation of STAT3, resistance to apoptosis and accelerated proliferation, resulting in enhanced hepatitis-induced hepatocarcinogenesis[64]. In addition, hepatitis C virus (HCV) core protein exerts an inhibitory effect on *(SOCS)-1* gene expression[65]. Hepatitis viruses also activate STAT-3 *via* oxidative stress[66-68], which might contribute to cellular transformation[69]. Abrogation of constitutive STAT3 activity sensitizes human hepatoma cells to apoptosis induced by TRAIL or drugs[70,71].

The PI3K/Akt pathway is also altered in HCC. The expression of the *PTEN* gene product is reduced or absent in almost half of HCCs and hepatocyte-specific abrogation of *PTEN* expression in mice results in the development of HCCs[72]. Recent results have indicated that the expression of a negative regulator of PI3K (phosphatidylinositol-3-kinase interacting protein I: PIK3IP1) is reduced in most cases of human HCC, pointing to a tumor suppressor-like function for this protein[73]. Interestingly, hepatic overexpression of PIK3IP1 negatively regulates PI3K activity in the tissue and suppresses the development of HCC[73].

Overexpression of Ras proteins is frequently observed in HCC[74], at least in part due to epigenetic silencing of inhibitors of the Ras pathway[75]. Furthermore, it has been reported that the expression of different ERK inhibitors, such as the Spred family of Ras/ERK inhibitors or the dual-specificity phosphatase-1 (DUSP1), is dysregulated in

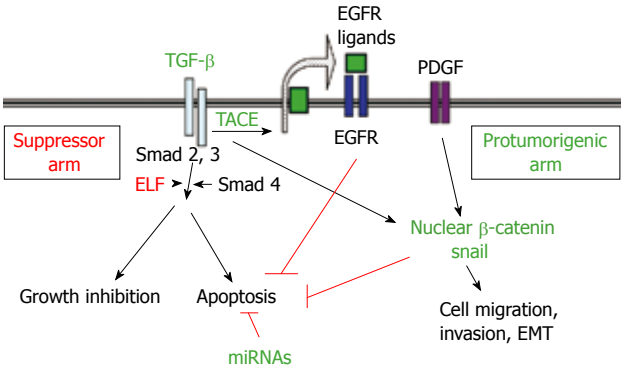

Figure 1 Dysregulation of the TGF-β pathways in HCC cells favours its pro-tumorigenic activities. In red, molecules whose expression is down-regulated; in green, molecules either up-regulated or overactivated. See text for details.

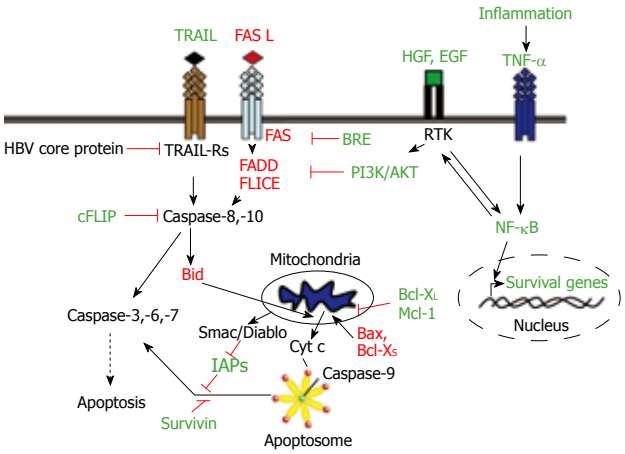

Figure 2 Alterations in the expression or functions of death receptor pathways and apoptosis regulatory proteins in HCC cells. In red, proteins either down-regulated or inactivated; in green, proteins either up-regulated or overactivated. See text for details.

HCC[76,77]. Activated *RAS* oncogene collaborates with the hepatitis B virus HBx protein to transform cells by suppressing HBx-mediated apoptosis[78]. Thus, dysregulation of the Ras pathway might also be playing a role in balancing pre-neoplastic hepatocytes towards survival in HBV- or HCV-mediated HCC.

In summary, different molecular alterations may contribute to an enhancement of anti-apoptotic signals in HCC cells that allow them to survive pro-apoptotic stimuli (Figure 3).

LIVER INFLAMMATION AND RESISTANCE TO APOPTOSIS

A link between inflammation and liver cancer was suspected some years ago, but the precise mechanisms are just beginning to be understood[79]. Recent experimental data support the hypothesis that inflammation promotes carcinogenesis and that NF-κB signalling is at the heart of such inflammation[79]. Different studies have implicated members of the NF-κB/Rel family in both HBV- and HCV-induced neoplastic development of the liver[80].

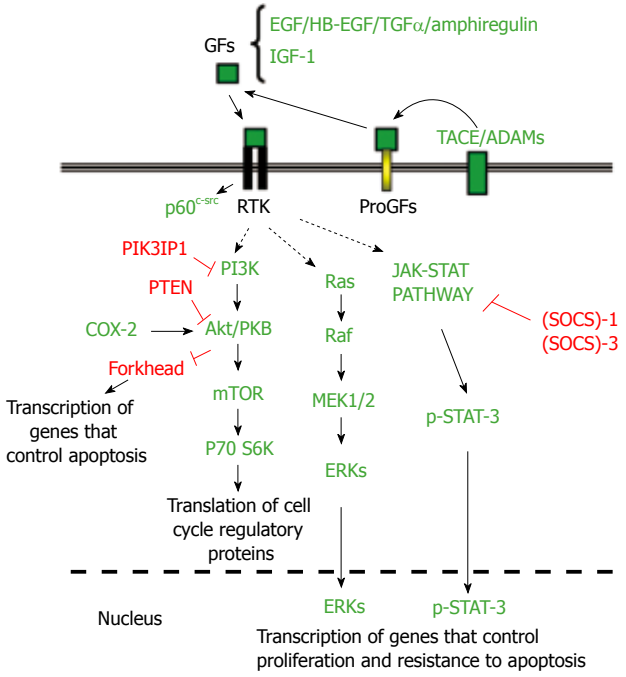

Figure 3 Overactivation of survival signals in HCC cells. In red, proteins either down-regulated or inactivated; in green, proteins either up-regulated or overactivated. See text for details.

Several mechanisms have been proposed for activation of NF-κB by the hepatitis virus. Overall, inflammatory hepatitis might activate NF-κB by the concerted action of cytokines, such as TNF-α, chemokines or interleukins, and viral proteins, which likely will promote cell survival of pre-cancerous hepatocytes[80]. Furthermore, a correlation between EGFR ligands and NF-κB activity has been provided by studies in transforming growth factor-alpha (TGF-α)/c-Myc mice. Indeed, an important role for NF-κB in inhibiting c-Myc-induced apoptosis was found essential for hepatocarcinogenesis[81]. Two pro-survival NF-κB targets are an antiapoptotic member of the Bcl-2 family, Bcl-XL, and a member of the caspase inhibitors, XIAP, which are frequently overexpressed in human HCCs, as commented above[44,48]. Interestingly, the NF-κB/Bcl-XL/XIAP axis potently counteracts the TGF-β-induced apoptosis[82] and exerts a general cytoprotective role on preneoplastic hepatocytes[83]. Recent results also link NF-κB to the increase in the autocrine expression of EGF receptor ligands, such as TGF-α, in hepatocytes and hepatoma cells[84,85]. In summary, overactivation of the NF-κB pathway might generate resistance to apoptosis, through different mechanisms, in HCC cells (Figure 2).

Many epidemiological studies demonstrate that treatment with non-steroidal anti-inflammatory drugs (NSAIDs) reduces the incidence and mortality of certain malignancies, especially gastrointestinal cancer[86]. The cyclooxygenase (COX) enzymes are well known targets of NSAIDs. Overexpression of COX-2 in HCC cells increases proliferation and survival through Akt activation[87]. Accordingly, recent evidence indicates that selective inhibition of COX-2 in HCC cells leads to a marked induction of apoptosis and inhibition of proliferation and, thus, may offer therapeutic and preventive poten-

tial in human hepatocarcinogenesis[88]. COX-2 inhibitors might induce apoptosis signalling in HCC cells via death receptors and mitochondria[89]. Recent data have demonstrated that simultaneous inhibition of PI3K/Akt/mTOR and COX-2 activity in *in vitro* models causes massive apoptosis of neoplastic hepatocytes[90].

EPITHELIAL-MESENCHYMAL TRANSITIONS AND APOPTOSIS RESISTANCE

During later stages in the development of liver tumors, a loss in cell-cell contacts and the acquisition of fibroblastic-like phenotype is observed. This phenomenon, known as epithelial-to-mesenchymal transition (EMT), might contribute to increasing the migratory and metastatic capabilities of the cells[91]. Cytokines, such as TGF-β and extracellular matrix molecules are thought to fundamentally contribute to the microenvironmental interaction between stromal and malignant cells, and provide the basis for a broad repertoire of epithelial transdifferentiation. Interestingly, EMT of liver cells also results in enhanced resistance to apoptosis[92,93], probably due to up-regulation of *SNAI1*, the gene that codifies for Snail, a repressor of E-cadherin expression that also has effects on cell homeostasis, inhibiting the cell cycle and preventing cell death[94] (Figure 1).

A high percentage of human HCCs show high levels of β-catenin[95,96], either through stabilizing mutations of the β-catenin or overexpression of *FZD*, therefore favouring the intracellular accumulation of the protein[95]. Furthermore, certain evidence indicates that TGF-β might induce nuclear β-catenin accumulation, through induction of PDGF signalling[97] (Figure 1). β-catenin expression leads to elevated EGFR levels in hepatocytes and inmunohistological analysis shows high correlation between the expression of nuclear/cytoplasmic β-catenin and EGFR in most hepatoblastomas[57]. β-catenin also participates in homotypic cell-cell interactions through its association with E-cadherin. Thus, β-catenin accumulation in HCC cells might contribute to impairing E-cadherin expression, mediating the EMT process, migration and survival. Indeed, there is evidence suggesting that up-regulation of *CTNNB1*, the gene encoding for β-catenin, also contributes to the enhancement of hepatocellular carcinoma cell survival[98].

In summary, a significant number of relevant molecular mechanisms altered in HCC initiation and progression are compromising the balance between survival and apoptotic signals in the pre-neoplastic hepatocytes. Some physiological pro-apoptotic molecules are down-regulated or inactivated in HCC, but the balance between death and survival is mainly disrupted due to overactivation of anti-apoptotic signals. Therefore, liver cancer cells might show stronger requirements of these intracellular pathways to survive. The absence of standard systemic therapy for advanced cases of HCC has changed with the recent positive randomized trial testing the multikinase sorafenib, which represents a breakthrough in the management of this neoplasm[3,58]. Interestingly, sorafenib induces tumor cell apoptosis in HCC cells, through, at the least, inhibiting the RAF/MEK/ERK pathway[99]. Similar situations might be found with other multikinase inhibitor drugs that are on the way towards approval for HCC therapy[58,100]. Of relevance here is certain evidence indicating that erlotinib-induced growth inhibition in HCC cells correlates with overexpression of pro-apoptotic factors like caspase and gadds, as well as down-regulation of anti-apoptotic factors, such as Bcl-X_L[101]. Another receptor tyrosine kinase inhibitor, sunitinib, which has also shown intriguing outcomes in advanced HCC[100], is a strong apoptosis inducer in different tumor cells, an effect that is enhanced in the presence of inhibitors of the PI3-K/Akt/mTOR pathway[102]. Bevacizumab, an anti-vascular endothelial growth factor (VEGF) monoclonal antibody, has been proven to be efficient in inhibiting the growth of nonmetastatic HCC[103]. Interestingly, recent evidence indicates that VEGF signalling inhibitors might be effective in inhibiting tumorigenesis more through their pro-apoptotic than their anti-angiogenic properties[104]. Therefore, therapeutic strategies to selectively inhibit anti-apoptotic signals in HCC cells might have the potential to provide powerful tools in the future to treat liver cancer.

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EDITORIAL

Do we really understand what the immunological disturbances in inflammatory bowel disease mean?

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Abstract

The gastrointestinal tract uses a system of tolerance and controlled inflammation to limit the response to dietary or bacteria-derived antigens in the gut. When this complex system breaks down, either by a chemical or pathogenic insult in a genetically predisposed individual the resulting immune response may lead to inflammatory bowel disease. Although the aetiopathogenesis of inflammatory bowel disease remains unsolved current evidence indicates that defective T-cell apoptosis and impairment of intestinal epithelial barrier function play important roles. In inflammatory bowel disease, it has been reported that activation of macrophages seems to be as important as increased production of the macrophage-derived cytokines such as TNF-α, IL-1 and IL-6. The triggering factor for this cascade is still to be elucidated as to whether it represents an auto-antigen or a hetero-antigen. It has been also demonstrated that a serologic anti-microbial response exists. This response includes antibodies against saccharomyces cerevisiae (ASCA), *E. coli* outer membrane porin C (Omp-C), flagelin (cBir1) and pseudomonas aeroginosa (I2). Host response to microbial pathogens includes self-defense mechanisms including defensins, pattern recognition receptors and Toll-like receptors. Neuroimmunomodulation in inflammatory bowel disease (IBD) is another interesting approach with implications on the influence of brain-gut axis on intestinal inflammation and its perpetuation. It is probable that inflammatory bowel disease represents a heterogenic group of diseases that share similar mechanisms of tissue damage but have different initiating events and immunoregulatory abnormalities. A better understanding of all these events will hopefully provide new insights into the mechanisms of epithelial responses to microorganisms and ideas for therapies.

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Key words: Inflammatory bowel disease; Immunological disturbances; Serological markers; Pathogenesis

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INTRODUCTION

Although the pathogenesis of inflammatory bowel disease (IBD) still remains unexplained, its pathophysiological mechanisms have been more extensively investigated and correspond to what is known about inflammatory disease processes in general. Our understanding of these disorders has benefited enormously from the development of novel animal models and recent advances in cell and molecular biology. Nowadays, it is commonplace that in IBD, a wide diversity of immunological changes occurs, including altered populations of inflammatory cells and activation of a range of inflammatory pathways.

TOLERANCE AND CONTROL OF INFLAMMATION IN IBD

The gastrointestinal tract uses a system of tolerance and controlled inflammation to limit the response to dietary or bacteria-derived antigens in the gut[1-3]. When this complex system breaks down, either by a chemical or pathogenic insult in a genetically predisposed individual

the resulting immune response may lead to IBD[4-8]. Although the aetiopathogenesis of IBD remains unsolved, current evidence indicates that defective T-cell apoptosis and impairment of intestinal epithelial barrier function play important roles[9,10]. Differences in T-cell responses between Crohn's disease (CD) and ulcerative colitis (UC) have been identified, with mucosal T-cell apoptosis being defective in CD but not in UC[7]. In both CD and UC, it has been reported that activation of macrophages seems to be as important as increased production of the macrophage derived cytokines TNF, IL-1 and IL-6[11,12].

Chemokines play a central role in the pathogenesis of IBD as they are able to trigger multiple inflammatory actions including leukocyte activation and chemoattraction, granulae exocytosis, production of metalloproteinases for matrix degradation and upregulation of the oxidative burst[13]. Dysregulated cytokine production by mucosal lymphocytes and macrophages has been implicated in the pathogenesis of IBD. In fact, an exclusive increase of CD4+ T cells in inflammatory bowel disease and their recruitment as intraepithelial lymphocytes has been demonstrated[14,15], while a potential therapeutic effect of capthesin inhibition *via* macrophages *in vivo* has been also suggested[16,17]. Over the past few years, various murine models of chronic intestinal inflammation resembling IBD have been discovered that have provided important clues as to the nature of this dysregulation and to its possible treatment with cytokines, for example IL-10[18,19].

It is clear from various studies that nuclear factor kappa B (NF-κB) and various other regulatory proteins are very likely to play a key role and it seems that we will gain new, fundamental insights into the pathogenesis, prognosis and treatment of IBD by analyzing further transcriptional regulatory mechanisms in chronic intestinal inflammation. This allows us to understand the basis for altered cytokine gene transcription in patients with IBD. Furthermore, such studies will hopefully permit the design of new treatment strategies that will be able to add specificity but reduced toxicity compared with standard immunosuppressive therapies[20].

TRIGGERING FACTORS OF THE INFLAMMATORY CASCADE IN IBD

The symptomatic phases of IBD are characterized by migration of large numbers of neutrophils and accumulation in the intestinal lumen. The triggering factor for this cascade is still to be elucidated whether it represents an auto-antigen or a hetero-antigen, i.e. a microbial component. Dysbiosis is the disturbance of intestinal microflora resulting in the breakdown in the balance between 'protective' *vs* 'harmful' intestinal bacteria[21]. Dysbiosis is implicated in many chronic diseases such IBD, which are associated with 'westernized' life style. It has been shown that enteric bacteria do not have equal capacities to induce or protect from inflammation and, interestingly, even *H pylori* infection has been implicated in CD pathogenesis[22]. It has been also demonstrated that in CD patients a serologic 'anti-microbial' response exists. In fact, granulomas in mesenteric lymph nodes from CD patients are composed of centrally located T-lymphocytes and of epithelioid cells, which are of monocyte/macrophage origin and have the characteristics of antigen presenting cells[23].

Currently available 'anti-microbial' response panel of laboratory tests includes antibodies against saccharomyces cerevisiae (ASCA), *E. coli* outer membrane porin C (Omp-C), flagelin (cBir1) and pseudomonas aeroginosa (I2)[24-26]. Of particular interest is that ASCA may develop before the obvious clinical diagnosis of CD according to a study using serum samples from the Israeli Defense Corp repository. In this study, 32% of patients were ASCA (+) 38 mo before CD clinical diagnosis was established[27]. The target antigen for pANCA is currently unknown and there is still variation regarding the inter-observer agreement with the several assays used for their determination. These serologic markers may be of great potential importance as they can provide more information on the IBD pathogenesis, the differentiation between UC and CD, the definite diagnosis of indeterminate colitis cases, the prediction of pouchitis and prediction of response to therapies. It is of importance that ASCA have a genetically modulated expression as they are found in 20%-25% of relatives of CD patients and are absent in spouses. In addition, pANCA are present in up to 20% of unaffected relatives of UC patients and they persist after colectomy indicating into two points: that the target antigen in IBD is not fully eradicated and that it is not just the colon which is immunologically targeted in UC [28-31].

It has to be emphasized that the serological markers are far from being the gold standard in IBD diagnostics and need to be combined, as they do not work separately in the clinical setting.

HOST RESPONSE TO MICROBIAL PATHOGENES

Host response to microbial pathogens includes self-defense mechanisms such as defensins, pattern recognition receptors (PRRs), pathogen-associated molecular patterns (PAMPs) and Toll-like receptors (TLRs). Toll-like receptors recognize conserved motifs on pathogens that are not found in higher eukaryotes and initiate 'innate' (rapid and non-specific) immune response. Subsequently, specific receptors recognizing chemo-attractant molecules mobilize phagocytic leukocytes and induce their migration to inflammatory sites. There, leukocytes encounter the invading microorganisms and ingest them through the activation of phagocytic receptors that mediate the uptake process. Innate immune responses are linked to the generation of corresponding adaptive immune responses and studies of genetically engineered or cellularly manipulated animal models have generated a great deal of new information[32].

Leukocyte-epithelial interactions are of special inter-

est as exposure of epithelial TLRs to microbial ligands has been shown to result in transcriptional upregulation of inflammatory mediators whereas ligation of leukocyte TLRs modulate specific antimicrobial responses[26]. It has been shown that Paneth cells play an important role in innate host defense *via* their ability to secrete antimicrobial peptides and proteins[33]. In addition, it has been shown that NOD2 mutations lead to loss of negative regulatory effects on TLR signaling while activation of the CARD domain results in activation of NF-κB[34].

THE MOLECULAR BASIS OF THE INTESTINAL IMMUNITY

Major advances in our understanding of the molecular basis of Rho guanine-triphosphatases (GTPases) function in regulating the phagocytic leukocytes that constitute the innate immune response have also been made. However, significant challenges remain. The molecular mechanisms involved in sensing chemotactic gradients, in maintaining polarized movement, and in coordinating the dynamics of the actin, myosin and microtubule cytoskeletons that are mediated by Rho GTPases remain to be worked out[32].

Matrix metalloproteinases (MMPs) seem also to play a crucial role in physiological and pathophysiological reactions such as leukocyte accumulation into inflamed tissue, cytokine production from inflammatory and epithelial cells, T lymphocyte homing to the intestine, wound healing and proliferation of epithelial cells, and intestinal innate immunity and permeability[35].

Neuroimmunomodulation in IBD remains a challenging theory with implications on the influence of brain-gut axis on intestinal inflammation and its perpetuation[36-38]. In recent years, considerable evidence has accumulated that psychological stress does indeed contribute to the risk of relapse in IBD. Furthermore, laboratory research has indicated a variety of mechanisms by which stress can affect both the systemic and gastrointestinal immune and inflammatory responses[39].

UNDERSTANDING THE MULTIFACTORIAL CHARACTER OF IBD

Despite decades of research the etiology of IBD remains largely unexplained, even though there is agreement with regard to these disorders' multifactorial character. Considering the epidemiological, genetic and immunological data, we can conclude that IBD are heterogeneous disorders of multifactorial etiology in which hereditary (genetic) and environmental (microbial, behavior) factors interact to produce the disease. It is probable that patients have a genetic predisposition for the development of the disease coupled with disturbances in immunoregulation. The disease can then be triggered by any of a number of different unknown environmental factors and sustained by an abnormal immune response to these factors. Rather, the intensive interaction between intestinal epithelial cells and immune competent cells is critical to maintain and perpetuate the chronic inflammatory process characteristic for IBD.

Once the role of genetic determinants is fully understood, early interventions can be designed to prevent disease in predisposed individuals. Gene therapy or modification of bacterial flora with probiotics or antibiotics or both for specific pathogens is expected to be central to this approach. From population-based data, important hypotheses for more specialised studies can be created. Especially if environmental influences are suspected in the onset and/or cause of a disease, population-based studies are indispensable[40]. However, good population-based studies are rare, probably because of the difficulties in organization and the long-term engagement needed[41].

Regarding the role of environmental factors, Hippocrates wrote in his "Epidemics"[42] that "patients with abscesses without fever, as well as patients with bloody stools became sick because of the same reason: at younger age they used to live under very difficult conditions and they were forced to use their body energy and muscular power to survive. However, later on, by becoming older they turned to work less harder, their body weight increased dramatically and their flesh became soft and vulnerable."

Parallel to Hippocratic skepticism, forty years ago Burrill B. Crohn[43] asked himself a rhetoric question: "Are these inflammatory bowel diseases the product of our modern civilization, the end product of industrial revolution? The answer is not evident."

THE PRESENT AND THE FUTURE OF THERAPY IN IBD

Today we target different immunological mechanisms and we use basically two groups of treatment: immunosuppressives and anti-inflammatory molecules.

The target point(s) of the currently used immunosuppressives is still unclear. However it has been suggested that azathioprine has a direct effect on the leukocyte nucleus and migration ability towards the site of inflammation[44] while methotrexate acts as an antimetabolite of the folic acid[45].

The anti-inflammatory therapies are various depending on the mechanism designed to hit: proinflammatory cytokines inhibitors (i.e. anti-TNF-α therapies), anti-inflammatory cytokine mediators (IL-10, IL-11), adhesion molecule inhibitors (i.e. anti-a4 integrin monoclonal antibody), T-cell inhibitors (anti-CD3 monoclonal antibody), cell based therapies (i.e. absorption aphaeresis), signal transduction inhibitors, transcription factor inhibitors and hematopoietic growth factors[46].

All physicians caring for patients with inflammatory bowel disease have an expanding arsenal of medications that can achieve symptomatic remission and mucosal healing. Adding biologics to existing immunomodulators improves our rates of sustained remission and healing of mucosal ulcerations. However, further studies are

needed to help determine what our final therapeutic endpoint should be.

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EDITORIAL

Clinical analysis of primary anaplastic carcinoma of the small intestine

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Abstract

Primary anaplastic carcinoma is a rare variant of small intestinal cancer. Most reports of primary anaplastic carcinoma of the small intestine are isolated case reports, therefore the clinicopathological features, therapeutic management, and surgical outcome of this tumor type remain unclear. This review analyzes the available clinical characteristics of primary anaplastic carcinoma of the small intestine and investigates key differences from differentiated adenocarcinoma of the small intestine. A Medline search was performed using the keywords 'small intestine' and 'anaplastic carcinoma' or 'undifferentiated carcinoma'. Additional articles were obtained from references within the papers identified by the Medline search. The literature revealed a poor prognosis for patients who underwent surgical resection for anaplastic carcinoma of the small intestine, which gave a 3-year overall survival rate of 10.8% and a median survival time of 5.0 mo. The literature suggests that anaplastic carcinoma is markedly more aggressive than differentiated adenocarcinoma of the small intestine. Surgical resection with the aim of complete tumor removal provides the only beneficial therapeutic option for patients with anaplastic carcinoma of the small intestine, because chemotherapy and radiation therapy have no significant effect on the rate of survival. However, despite complete tumor resection, most patients with anaplastic carcinoma of the small intestine are at great risk of disease recurrence. Multicenter clinical trials are expected to provide additional therapeutic strategies and establish the efficacy of multimodality adjuvant therapy. This report also highlights the importance of a systematic diagnostic approach for anaplastic carcinoma of the small intestine.

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Key words: Anaplastic carcinoma; Small intestinal cancer; Small intestinal tumor; Operation; Prognosis

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INTRODUCTION

The small intestine represents more than 75% of the length and 90% of the total mucosal surface area of the alimentary tract; however, it contributes only about 1% of malignant gastrointestinal tumors[1]. Several hypotheses have been proposed to explain why small intestinal tumors are rare[2]. While there are many reported reviews of small intestinal tumors[3-7] including sarcomas[8,9], only a few describe the occurrence of small intestinal carcinoma[10-13]. Small intestinal carcinomas are uncommon among the malignant tumors that occur throughout the entire alimentary tract but represent 50% of small intestinal malignant tumors. Anaplastic carcinoma is a rare variant of small intestinal carcinoma. Most reports describing the occurrence of anaplastic carcinoma of the small intestine do not include a detailed description of the pathology of this disease. The aim of the present study was to review the clinical presentation, surgical management, and prognostic factors of primary anaplastic carcinoma of the small intestine.

PATIENTS AND CLINICAL PRESENTATION

We reviewed 18 patients who underwent surgical resection for anaplastic carcinoma of the small

Table 1 Clinicopathological data for reported cases of anaplastic carcinoma of the small intestine

Case	Author	Yr	Age	Gender	Chief complaint	Primary site	Tumor size (cm)	Tumor depth	Lymph node metastasis	Prognosis (mo)
1	Ikeda[22]	1974	62	F	Abdominal pain	Ileum	9	SE	Negative	Alive 12
2	Sasaki[23]	1988	37	M	Abdominal pain	Jejunum	7	SS	Positive	9
3	Robey-Cafferty[24]	1989	62	M	Cervical LN enlarge	Ileum	5	SI	ND	20
4			38	F	Fatigue	Jejunum	16	MP	Positive	8
5			48	F	Periumbilical mass	Jejunum	6	SI	Positive	2
6			65	M	Abdominal pain	Jejunum	5	SE	Positive	5
7			54	F	intestinal obstruction	Ileum	4.5	MP	Negative	Alive 12
8			35	F	Abdominal pain	Jejunum	7.5	ND	Negative	36
9	Ikeguchi[25]	1993	63	M	Abdominal pain	Ileum	4	SS	Negative	Alive 36
10	Amano[26]	1998	81	M	Abdominal pain	Jejunum	8.5	SE	Positive	1
11	Agrawal[27]	1999	53	M	melena	Ileum	13	SI	Positive	2
12	Nakamura[28]	2000	73	F	Vomiting	Ileum	3.5	SS	Negative	6
13	Kadoya[29]	2003	57	M	General fatigue	Ileum	21	SE	Positive	2
14	Sato[30]	2003	46	M	Back pain	Jejunum	9	SE	ND	4
15	Usuda[31]	2007	84	M	Fever of unknown origin	Jejunum	8	SE	Positive	1.5
16	Shiraishi[32]	2007	48	M	Abdominal pain	Jejunum	5	SE	Negative	Alive 8
17	Matsuoka[33]	2008	56	M	Abdominal pain	Jejunum	10	SS	Positive	1
18	Our case	2008	65	F	Anemia	Ileum	6	SS	Negative	Alive 8

MP: Muscularis propria layer; SS: Subserosal layer, SE: Penetration of serosa; SI: Invasion of adjacent structures; ND: Not described.

intestine between 1974 and 2008. Seventeen cases were identified in the available literature using a Medline search and Japan Centra Revuo Medicina (Table 1). One reported case was a patient treated in our hospital. The clinicopathological features of the 18 reported cases are listed in Table 1. The mean age of patients with anaplastic carcinoma of the small intestine was 58 years (range, 38-81 years). There was a slight male predominance, with a male-to-female ratio of 11:7. The most common presenting signs and symptoms of small intestinal cancer were nausea, vomiting, abdominal pain, melena, weight loss, anemia, and palpable mass, none of which is pathognomonic for small intestinal tumors. The most common presenting complaint of patients with anaplastic carcinoma of the small intestine was abdominal pain, which was present in 47% of patients. For malignant small bowel neoplasms the most common symptom was pain followed by gastrointestinal bleeding, weight loss, nausea, and vomiting. The nonspecific nature of the reported symptoms usually leads to a delay in presentation and work-up. The average diameter of tumors was 8.3 cm (range, 3.5-21 cm).

The most common location for small intestinal carcinoma is the duodenum[4]. More distal tumors are found more frequently in the jejunum than in the ileum[4,12]. By comparison, anaplastic carcinoma of the small intestine was not found in the duodenum but in the jejunum in 10 cases and in the ileum in 8 cases. The symptoms associated with primary small intestinal tumors were usually vague and nonspecific. The nonspecificity of the symptoms associated with this disease is considered a contributing factor in the delayed diagnosis associated with small intestinal tumors.

The pathogenesis of small intestinal carcinoma is largely unknown. Small intestinal regional enteritis, or Crohn's disease, is reported to be associated with small intestinal carcinoma. It is estimated that a carcinoma will develop in 2%-3% of patients with small intestinal regional enteritis[14]. One investigator reported a linear correlation between the incidence of small intestinal cancer and colon cancer[15].

Survival analyses of patients with small intestinal carcinoma were performed using the Kaplan Meier method with the log rank test to assess statistical significance. Values were expressed appropriately as the mean ± SD. The Cox proportional hazards regression model was used to assess the combined effects of covariates on survival. *P* values of less than 0.05 were considered significant.

TREATMENT OF SMALL INTESTINAL CANCER

In most studies, surgery as a curative treatment option for small intestinal cancer was performed in 40%-65% of patients, with reported 5-year survival rates of 40%-60% for resected tumors *versus* 15%-30% for nonresected tumors[10,11,16]. Many reports indicated that chemotherapy and radiation therapy did not significantly influence survival[12,17]. According to published reports, jejunal or ileal adenocarcinoma should be treated aggressively with segmental resection and primary anastomosis[18]. Pancreaticoduodenectomy with complete resection of the tumor and negative margins is associated with favorable long-term survival[19]. On the basis of this finding, patients with a tumor at the proximal duodenum should be treated by pancreaticoduodenectomy. Analysis of the findings of previous reports suggests that radical curative surgery is the most important prognostic factor for survival of patients with small intestinal cancer. Complete surgical resection has emerged as the most important prognostic variable in the management of this disease. A similar management strategy can be applied for the treatment of patients with anaplastic carcinoma

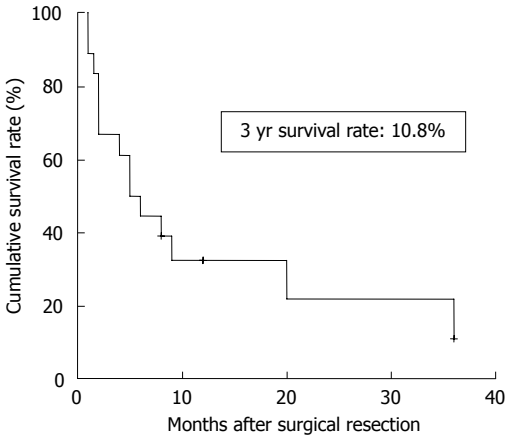

Figure 1 Survival after surgical resection for anaplastic carcinoma of the small intestine (*n* = 18).

of the small intestine. However, the prognosis for patients with small intestinal cancer is dismal.

PROGNOSTIC FACTORS OF RESECTABLE SMALL INTESTINAL CANCER

The 5-year survival rate for primary adenocarcinoma of the small intestine ranges from 17.5% to 37%[12,13,17]. In this review, the overall 3-year survival rate of anaplastic carcinoma of the small intestine after surgical resection was 10.8% (Figure 1). There were no patients who survived over 4 years. Thus, patients with anaplastic carcinoma of the small intestine have a poorer prognosis than patients with adenocarcinoma of the small intestine. In general, anaplastic carcinoma has a high malignancy grade potential. Consistent with findings reported in previous reviews, the prognostic factors that influence the survival of patients with anaplastic carcinoma include patient age, tumor site, clinical staging, curative resection surgery, tumor size, histological grade, nodal metastases, lymphangiosis carcinomatosa, and vascular invasion[6,12,17]. Univariate analysis of the different prognostic factors predicted to contribute to patient prognosis revealed that age and lymph node metastases were unfavorable prognostic factors. Overall median survival of patients over 65 years old with lymph node metastases was significantly worse than that for patients under 65 years old without lymph node metastases. Gender, tumor depth, tumor location, and tumor size were not significant prognostic factors (Table 2). By comparison, Veyrières *et al*[10] (1997) revealed the degree of tumor differentiation to be a significant prognostic factor. However, multivariate analysis demonstrated that lymph node metastases, and not tumor differentiation, was an independent prognostic factor[11]. For patients with anaplastic carcinoma of the small intestine, the median survival period was significantly shorter for patients with positive lymph nodes than for patients with negative lymph nodes, according to the Cox proportional hazards model (Table 3). When a curative surgical resection is performed before lymph node metastases, the chance of long-term survival is greatly increased. A previous study revealed a significant positive correlation between the survival of patients with adenocarcinoma of the small intestine and pathological differentiation (Table 4). In the present review, more patients with anaplastic carcinoma had a poorer prognosis than patients with adenocarcinoma, despite curative resection of the tumor. However, the number of patients is too small to allow an accurate evaluation of prognosis. The establishment of a multicenter registry would provide a basis for multivariate analysis of factors influencing survival.

Table 2 Clinical characteristics after surgical resection of anaplastic carcinoma of the small intestine (mean ± SD)

Characteristics	No. of patients	3 yr survival rate (%)	Median survival in months	*P* value
Overall	17	10.8	5.0 ± 1.4	
Age (yr)				
< 65	13	15	9.0 ± 4.2	0.03
> 65	5	0	5.0 ± 1.6	
Gender				
Male	11	12.1	4.0 ± 1.5	0.38
Female	7	0	8.0 ± 2.6	
Tumor depth				
T2 (MP)	2	66.7	26.7 ± 7.6	0.21
T3 (SS)	5	0	6.0 ± 1.5	
T4 (SE, SI)	10	0	2.0 ± 1.0	
Tumor location				
Jejunum	10	0	4.0 ± 2.4	0.17
Ileum	8	25	6.0 ± 7.0	
Tumor size (cm)				
< 7	8	28.6	20.0 ± 10.9	
> 7	10	0	2.0 ± 1.3	0.13
LN metastasis				
Negative	7	41.7	36.0 ± 21.9	0.0009
Positive	9	0	2.0 ± 0.3	

MP: Muscularis propria layer; SS: Subserosal layer; SE: Penetration of serosa; SI: Invasion of adjacent structures.

Table 3 Relative risk with regard to survival rate according to the Cox proportional hazards model

Variable	Odds ratio (95% CI)	*P* value
Lymph node metastasis		
Negative	13.7 (1.6-114.1)	0.016
Positive		
Age (yr)		
< 65	2.9 (0.7-11.8)	0.145
> 65		

METHODS AND EFFICACY OF EARLY DIAGNOSIS

The poor prognosis for anaplastic carcinoma of the small intestine may be related to a delay in the diagnosis and treatment of the disease. An early and accurate diagnosis is essential to improve the prognosis for patients with small intestinal cancer. The correct

Table 4 Survival rates of patients with adenocarcinoma of the small intestine according to pathological differentiation

Author	Differentiation	No. of patients	Survival rate (%)		Significance
			5 yr	10 yr	
Miles[7]	Well differentiated	13	15	8	0.18
	Undifferentiated	5	60	33	
Veyrieres[10]	Well differentiated	51	56	43	0.08
	Undifferentiated	6	40	0	
Dabaja[11]	Well differentiated	92	25		0.45
	Undifferentiated	57	24		
Wu[13]	Well differentiated	33	88.7 ± 18.88[1]		0.26
	Undifferentiated	10	13.3 ± 3.96[1]		
Robey-Cafferty[24]	Well differentiated	29	25		0.24
	Undifferentiated	6	0		

[1]Mean survival time (months ± SD).

diagnosis of small intestinal tumors remains difficult, because the tumors are rare and the symptoms are ambiguous. Performance of a conventional double-contrast series of the small intestine proved to be an excellent diagnostic modality, but is increasingly being replaced by cross-sectional imaging methods including computed tomography (CT) and magnetic resonance imaging (MRI). CT was the most efficient method for demonstrating the presence of a mass lesion, which suggests a locally advanced tumor with contiguous organ invasion, and for confirming the absence of distant metastases. Recent advances in multidetector CT (MDCT) technology have allowed the production of high-resolution cross-sectional images of the abdomen and the small intestine[20]. Upper gastrointestinal endoscopy is an accurate method for the diagnosis of duodenal tumors, but deep insertion of an endoscope by the push technique is difficult. A novel double-balloon enteroscopy system, which was developed by Yamamoto *et al*[21], has been applied for diagnosis in patients with obscure gastrointestinal bleeding or chronic abdominal pain. The double-balloon enteroscopy system has the advantage of allowing examination of the entire small intestine by direct visualization. It is apparent from these studies that early enteroscopic examination must be performed as soon as carcinoma of the small intestine is suspected, to allow accurate and early diagnosis.

CONCLUSION

Anaplastic carcinoma of the small intestine is an extremely rare disease, is often diagnosed at an advanced stage, and displays an aggressive clinical course. Despite treatment by means of optimized surgical procedures, patients who have undergone complete tumor resection have a poor prognosis. To improve the outcome of surgical treatment, additional therapy, such as multimodality adjuvant therapy or the administration of novel molecular targeted drugs, should be considered for patients with anaplastic carcinoma of the small intestine. Furthermore, randomized, prospective clinical trials need to be conducted to demonstrate an unequivocal survival advantage for patients who have received chemotherapy or radiation therapy after resection of anaplastic carcinoma of the small intestine.

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REVIEW

Bio-mathematical models of viral dynamics to tailor antiviral therapy in chronic viral hepatitis

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Abstract

The simulation of the dynamics of viral infections by mathematical equations has been applied successfully to the study of viral infections during antiviral therapy. Standard models applied to viral hepatitis describe the viral load decline in the first 2-4 wk of antiviral therapy, but do not adequately simulate the dynamics of viral infection for the following period. The hypothesis of a constant clearance rate of the infected cells provides an unrealistic estimation of the time necessary to reach the control or the clearance of hepatitis B virus (HBV)/hepatitis C virus (HCV) infection. To overcome the problem, we have developed a new multiphasic model in which the immune system activity is modulated by a negative feedback caused by the infected cells reduction, and alanine aminotransferase kinetics serve as a surrogate marker of infected-cell clearance. By this approach, we can compute the dynamics of infected cells during the whole treatment course, and find a good correlation between the number of infected cells at the end of therapy and the long-term virological response in patients with chronic hepatitis C. The new model successfully describes the HBV infection dynamics far beyond the third month of antiviral therapy under the assumption that the sum of infected and non-infected cells remains roughly constant during therapy, and both target and infected cells concur in the hepatocyte turnover. In clinical practice, these new models will allow the development of simulators of treatment response that will be used as an "automatic pilot" for tailoring antiviral therapy in chronic hepatitis B as well as chronic hepatitis C patients.

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Key words: Viral hepatitis; Bio-mathematical models; Hepatitis B virus; Hepatitis C virus; Viral dynamics

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INTRODUCTION

The standardization and application in clinical practice of quantitative assays of nucleic acids enable us to measure the quantity of virus or viral load in the blood (viremia), and to study its fluctuations during treatment with antivirals in patients with chronic viral hepatitis. Since viremia represents the equilibrium between virus production by the infected cells and virus clearance by the antiviral reaction of the immune system or host's antiviral immune response, viremia variations are the consequence of continuous alterations of the equilibrium between virus and host antiviral responses, which may vary during the natural course of the infection and are associated with the fluctuations of the liver disease activity[1,2]. However, the equilibrium between virus production and clearance can be artificially altered, which increases the clearance (removal) of circulating viruses (i.e. plasma-apheresis[3]), or reduces virus production with antiviral drugs. A rapid decline in viremia has been shown in patients with chronic human immunodeficiency viruses (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) infections a few hours after administration of antivirals[4-6]. We may study the mechanisms responsible for these viral kinetics using mathematical models that can interpret viremia fluctuations according to hypotheses based on biological knowledge. The simulation of the complex biological phenomena by means of mathematical equations based only on suppositions, hypotheses or biological evidence has progressed consistently in several fields of medicine during the past few decades. Such modeling provides a simplified description of the biological process, but it maintains sufficient complexity to uncover the major determinants of the process itself, by studying the fluctuations of measured variables and predicted parameters with several simulations.

STANDARD MODELS

In chronic viral infections, standard models consider the existence of two separate but linked compartments: in the former, viruses replicate inside the infected cells, and in the latter, free virions circulate in the blood (Figure 1).

Dynamics and relations among uninfected target cells (T), infected cells (I) and viral load (V) are described by three different equations. Briefly, these models assume that target liver cells (T) replicate at rate s, die at rate d, and become infected (I) at a certain rate (βTV) proportional to both T and V when they encounter free virions circulating in the blood. The infected cells are eliminated at the rate of δ I and free virions, which are produced at the rate of Ψ I, are cleared at the rate of λ V. The parameters d, β, δ, Ψ, λ are the specific constant rates of formation and clearance of infected cells and circulating free virions. Antiviral therapy can reduce target-cell sensitivity to infection or the production rate of free virions that are released in the blood from the already infected cells. The former mechanism was assigned originally to interferon (IFN) in cell cultures[7,8], and the latter to drugs that block viral replication, such as nucleos(t)ide analogs or protease inhibitors[9].

In this model, the impact of antiviral drugs on the dynamics of viral infection is considered by the coefficients (1-η) and (1-ε) of the equations that describe the rate of target cell infection and free virion production, where η and ε represent the reduction in cell susceptibility to virus infection and the reduction in free virions production, respectively (Figure 2). It is understood that the time required by the drug to achieve complete antiviral efficacy is quite short and much shorter that the circulating virion half-life.

The standard model predicts that the pattern of viral load decline in patients treated with potent antivirals that block virus production is biphasic if the half-life of the circulating virions ($T_{1/2} = \ln 2/\lambda$) and that of the infected cells ($T_{1/2} = \ln 2/\delta$) are different. The short half-life of the first phase of viral load decline suggests that it is associated with the clearance of circulating virions rather than infected cells (Figure 3).

Originally, Perelson *et al*[5], applying this approach to study HIV dynamics in patients treated with strong HIV-1 protease inhibitors, showed a very short half-life (nearly 6 h) of the virions in the blood and of infected cells (lymphocytes) (1.6 d)[5]. Likewise, when Neumann *et al*[6] used the same model approach in HCV-infected patients treated with IFN, they estimated that the virion half-life was 2.7 h. Moreover, contrary to HIV patients, they found that, just at the beginning of therapy, the estimated infected cell half-life (hepatocytes) was much longer (1.7-70 d), and was directly correlated with baseline viral load and inversely correlated with baseline alanine aminotransferase (ALT) levels.

With this analytical approach, the mechanism of IFN action in HCV patients is clear, and shows that the major antiviral effect is caused by a dose-dependent block of viral production in the infected cells. Therefore, the typical biphasic pattern of viral load decline observed in responder patients is to the result of a rapid reduction

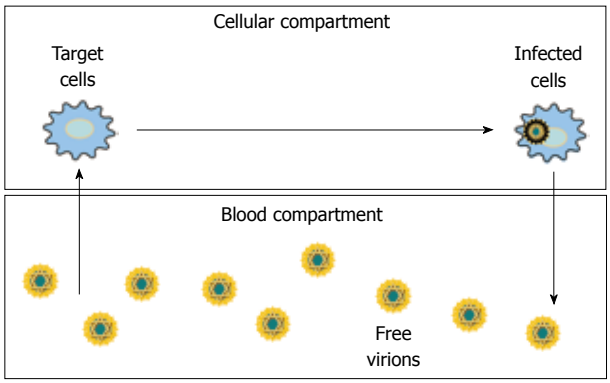

Figure 1 Two-compartment model.

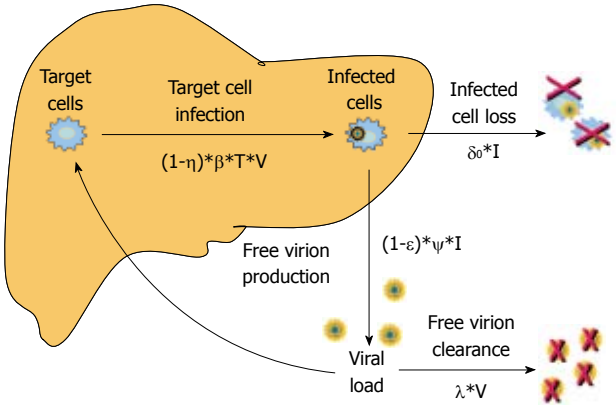

Figure 2 Basic model of viral dynamics.

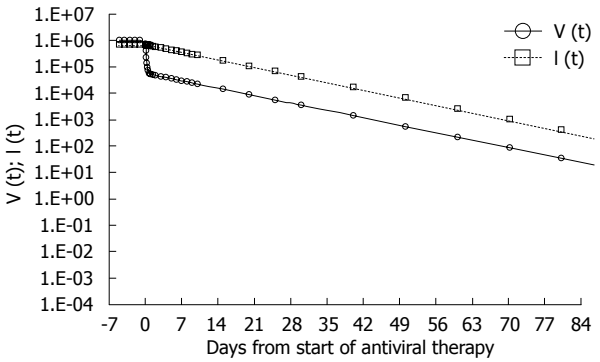

Figure 3 Predicted decline of viral load (V) and infected cells (I) in the basic model of viral dynamics.

of free virion production, followed by a slower decrease in the number of infected cells. The modeling approach suggests monitoring the effectiveness of therapy individually, using the viral load, and predicting carefully the antiviral treatment outcome, in order to tailor the therapy schedule[9,10]. On the contrary, the standard models, although describing well the viral load decline in the first 2-4 wk of therapy, cannot adequately simulate the dynamics of the infection for the following period of treatment. Thus, the standard models, with the hypothesis of a constant clearance rate of the infected cells, provide an unrealistic estimation of time necessary to reach control or clearance of HBV/HCV infection.

Table 1 Free virion and infected cell half lives computed by different models of HBV dynamics

Author	Therapy	HBeAg	Virion $T_{1/2}$ (h)	Inf. cell $T_{1/2}$ (d)	Antiviral effectiveness (%)
Nowak *et al*[13]	LMV 20-600 mg	POS	24	10-100	87-99
Lewin *et al*[16]	LMV 150 mg	POS	28.5	2.4 > 120	95
	LMV + FCV				99
Tsiang *et al*[14]	ADV	POS	26.4	11-30	99
Wolters *et al*[18]	LMV150 mg	POS	13[1]	< 0-331	92-96
Wolters *et al*[18]	ETV	POS/neg	16[1]	5.2-31.8	87-98
Sypsa *et al*[19]	PegIFN2b 1-200 mg	neg	12.7[1]	2.7-75	83
	LMV 100 mg				96
Colombatto *et al*[20]	PegIFN2a 180 mg	neg	nc	2.5-77.3	88
	PegIFN2a + LMV		8.2[1]	4.7-33.3	99.6 (87)
	LMV 100 mg		9.5[1]	4.3-56.5	99.4 (88)

[1]Tighter sampling schedule during the first 48 h.

MULTIPHASIC VIRAL DYNAMICS MODEL

To bypass this inconvenience, we have developed a multiphasic viral dynamics model in which we suppose a modulation of the immune system during therapy, according to negative feedback caused by a reduction in the number of infected cells. In addition, we utilized the ALT kinetics as a surrogate marker of infected-cell clearance. By this approach, we can compute the dynamics of infected cells during the whole treatment course, and we can find a good correlation between the infected cells number computed at the end of the therapy and the long term virological response in patients with chronic hepatitis C[11,12].

Concerning HBV infection, in 1996, Nowak *et al*[13] applied the standard model to analyze the decrease in viral load in patients treated with lamivudine (LMV), the first drug to show strong antiviral action on hepatitis B e antigen (HBeAg)-positive chronic hepatitis B. LMV is able to reduce significantly new viral production by infected cells and inihibit viral reverse transcriptase (RT)/polymerase. During the first rapid phase of viral load decline until the second day of treatment, Nowak *et al*[13] estimated the virus decline constant rate (u) at 0.67/d, which corresponded to a half-life of nearly 1 d for free virions. Moreover, seeing the slowing down of viral load decline after a few days of treatment, the authors reached the conclusion that the drug might have blocked only partially virus production by infected cells. Thus, to show the partial efficacy of the drug, they introduced a new factor, ρ, defined as ε in following studies, and, to explain the second phase decline of the viral load, they hypothesized that this phase may be related to the decline in the number of infected cells. Accordingly, Nowak *et al*[13] estimated with different approaches the infected cell clearance rate, by comparing viral production rate before and after therapy, and by analyzing the decline in HBeAg, an HBV secretory protein that is produced continuously by infected cells during LMV therapy. By these two methods, it was estimated that the infected cells' half-lives were equivalent and showed a wide range from 10 to 100 d (on average 16 d).

Another approach to the HBV model was implemented by Tsiang *et al*[14], who discarded the hypothesis that the number of infected cells is constant during therapy. They introduced the possibility that nucleos(t)ide analogs interfere with *de novo* infection of target hepatocytes since the RT/polymerase activity hampers the completion of the double-stranded DNA before migration towards the just-infected cell nucleous[15]. They have suggested that such an antiviral effect reduces the number of infected cells during treatment, and by this assumption, they were able to detail the HBV-DNA kinetics for 12 wk in patients treated with 30 mg/d adefovir (ADV)[14]. Using this model, Tsiang *et al*[14] have been able to show that the loss of infected hepatocytes is a rather slow process that can be described only from the second phase of viral load decline. They have reported half-lives for free virions and infected hepatocytes of 1.1 and 18 d, respectively, similar to those calcuated previously by Nowak *et al*[13].

Lewin *et al*[16], 2 years later, proposed instead a new model that suggests the possibility that infected cells can revert to their uninfected state after losing covalently closed circular DNA (cccDNA) by a non-cytolytic endogenous antiviral mechanism, similar to the one applied in the experimental models of acute HBV infection[17]. The authors have suggested that LMV or famciclovir (FCV) can partially inhibit new infections since cell polymerases in the hepatocytes nuclei can transform the circular HBV-DNA into cccDNA, which represents HBV matrix transcription. They have also found higher levels of variability in half-lives of free virions (from 1 to 92 h) and infected cells (from 2 to > 120 d). This variability is explained by the fact that even if most of the patients show a typical biphasic profile, the others show complex viral decline with "staircase" or multiphasic patterns.

Some of these patients, after the rapid first phase decline, had steady HBV-DNA levels for several days (even 4 wk) before viremia decreased, or in some cases, stabilized again. Variability in viremia decline may be explained by the patients' heterogeneity according to their different conditions of HBV infection. In fact, a phase in which viremia remains stable may depend on a patient's immunological condition, in which the infected cell clearance is very poor. This has been observed in patients at an early phase of HBV immune activation, which assumes a very low immune activation (very low δ) and a baseline number of infected cells approaching 100%. Wolters *et al*[18], following this interpretation, have

shown that higher baseline ALT levels are significantly associated with a greater rapidity of viral load decline in the second phase.

Different profiles of viremia decline may be caused by many reasons: modulation during therapy of cytolytic and non-cytolytic mechanisms of infected cell loss; presence of two or more infected cell populations with different half-lives; and infected cells with heterogeneity in their expression of drug-efflux pumps[16].

Lewin *et al*[16] have emphasized the complexity of HBV dynamics for treatments longer than a few weeks. Moreover, they have demonstrated the need for tight sampling immediately after drug administration, to warrant an accurate definition of viral clearance rate, and the need to evaluate the early stages of the delay before the drug starts its effect, as shown in HCV and HIV infections[5]. With tight viremia monitoring soon after the beginning of therapy (every 6 h in the first 2 d, compared to the 1-d interval adopted in previous studies), Wolters *et al*[18] have shown that the virion half-life is shorter (mean 15 h) than previously stated (24 h). These findings have been confirmed recently by other studies using higher sampling frequencies in the first 2 d of therapy (Table 1)[19,20].

In one of these studies, Sypsa *et al*[19] analyzed HBV dynamics in HBeAg-negative patients during the first 4 wk of treatment with pegylated IFN-2b (PegIFN-2b) monotherapy or combined with LMV. The standard model was applied to patients receiving LMV alone or in combination, and accounted for the decreasing effectiveness of the drug between the weekly doses to patients treated with PegIFN-2b monotherapy. In this model, the impact of antiviral drugs on the dynamics of viral infection is considered by the terms (1-η) and (1-ε) of the equations 1, 2 and 3 (Figure 2), where η and ε represent the reduction in cell susceptibility to virus infection and the reduction in free virion production. This holds that the time required by the drug to achieve complete antiviral efficacy is quite short and much shorter than the circulating virion half-life. In this way, they could observe the fluctuations of viral load across the week of treatment in some patients, and this was ascribed to the decline of drug concentration between dosages. Besides the dependence of ε from the PegIFN 2b concentration, the authors suppose that the number of target and infected cells remain constant during the period of analysis (4 wk) and for the first 7 d of treatment. Starting from these assumptions, they emphasized that the mean antiviral efficacy of LMV monotherapy was superior to that of PegIFN-2b monotherapy (96.4% *vs* 82.6%) and comparable to that of PegIFN-2b + LMV (92.8%-94.4% for 100 μg or 200 μg, respectively). Moreover, they found that the infected cell half-life was comparable among the various groups of treatment (9.8 d *vs* 5.0 d *vs* 6.0 d for PegIFN-2b monotherapy, combination or LMV monotherapy, respectively), ranging from 2.7 to 75 d. Standard modeling of HBV dynamics was limited to the first 4-12 wk of therapy and was used to study direct antiviral activity of the drug. Instead, when using the model to tailor the treatment, it is mandatory to simulate viral dynamics throughout the whole treatment course, and this means introducing additional hypotheses about hepatocyte proliferation and immune system activity after the first month of therapy.

The hypothesis that the infected cell clearance activity by the immune system remains constant during therapy is in contrast with some common observations in HBV infection. For example, IgM anti-HBc levels, as surrogate markers of HBV-induced liver damage, decline progressively during treatment[1,2]. Thus, we have to hypothesize that the decrease in infected cells during treatment leads to a reduced immune stimulation that, in turn, could determine a decrease in the clearance of infected cells. It is likely that the kinetics of viral load decline during the first month of treatment are not simply biphasic in a significant proportion of patients treated either with LMV or PegIFNs + LMV. The average slope of weekly viral load declines observed by Sypsa *et al*[19] was similar during days 14-21 and 21-28 (-0.027 and 0.033 log10 cp/mL per day, respectively), but slower than those observed during days 7-14 (-0.067 log10 cp/mL per day).

These findings were ascribed to the prolonged first phase decline of viral load in some patients. In fact, we highlighted that all patients treated with LMV or Peg2a + LMV[20] had a viral load decline characterized by a rapid drop during the first phase of therapy (average HBV-DNA half-life of about 10 h during days 0-4), followed by another rapid drop (average HBV-DNA half-life of about 2 d) between days 4 and 14 (in particular between days 4-7). Attributing this second phase to the reduction in infected cells, according to the biphasic model, the infected cell half-life would be about 2 d only, and this would mean that chronic hepatitis patients with about 50% infected hepatocytes could lose > 10% of their overall hepatic mass every day. We could overcome this criticism if we suppose that the virion production in these patients was really inhibited in two phases: firstly a sharp drop as described in the model by the term "1-ε", followed by a second slower drop with exponential decline.

It is evident from all these studies that investigation of the long-term dynamics of HBV infection during the whole course of the antiviral therapy needs more specific models that take into account the complexity of HBV biology and its interplay with the host's immune system. In consideration of the limitations previously described we developed a new model with simple biological assumptions on target and infected hepatocyte dynamics to make possible the simulation of the antiviral effects during the whole course of different antiviral treatments[20]. Briefly, the model holds that: (1) the sum of target and infected cells remains roughly constant and equals the total hepatocyte number in the normal liver ($H_0 = 2.5 \times 10^{11}$ cells); (2) infected hepatocytes can generate infected daughter cells, as shown in animal models[21]; and (3) both target and infected cells contribute to the hepatocyte turnover in proportion to their relative numbers.

The following equation describes the full dynamics of the infected hepatocytes during therapy: $dI(t)/dt =$

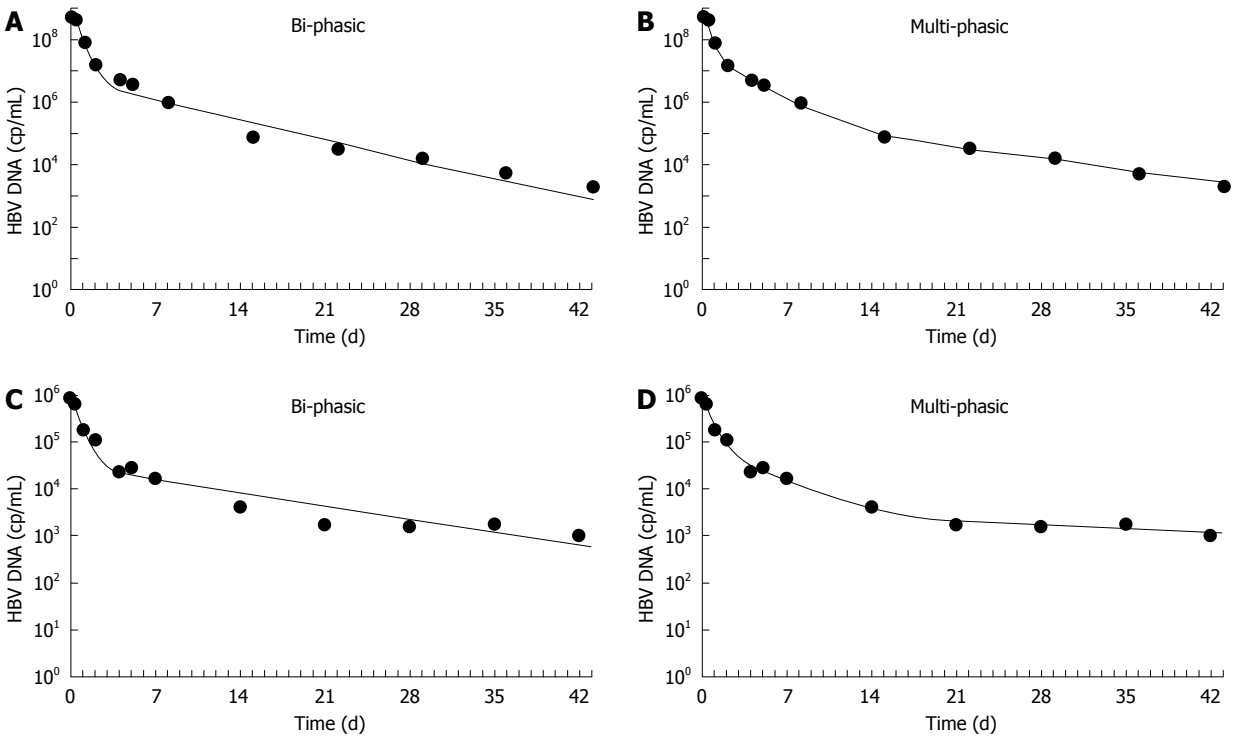

Figure 4 Biphasic *versus* multiphasic fitting of the early HBV-DNA decline in patients treated with LMV.

$(1-\eta)\beta \cdot T(t) \cdot V(t) - \delta(t) \cdot I(t) \cdot [1-I(t)/H_0]$. where η takes into account any variation of the susceptibility of target cells to get infected, as induced by therapy.

ALT serum levels as a marker of hepatocyte damage[22], as in other models[11,23], were used to determine the infected hepatocyte number at baseline, according to the following equation: $I_0 = [ALT_0-20]/[\Phi \cdot \delta_0]$, where the numerical value of the constant Φ is equal to 3.24×10^{-4}[11], calculated under the assumption that the normal ALT value is equal to 20 U/L and the natural hepatocyte turnover is 300/d[24].

The new model integrated the above hypothesis that the clearance rate of infected cells in HBeAg-negative chronic hepatitis patients could decrease after the first month of therapy because of the negative feedback derived from the progressive reduction of infected hepatocytes. Thus the equation describing the immune system activity was changed to: $\delta(t) = \delta_0 \cdot [I(t-\sigma)/I_0]^k$ where σ is the time elapsing between the variation in the number of infected cells and the variation in the immune system activity (that is assumed to be 35 d), and k (which can vary from 0 to 1), which is the feedback parameter that links the variation in the virus-specific immune clearance activity to the variation in the number of infected cells. The multi-phase profile of the viral load decline observed in patients treated with LMV or Peg-IFNs + LMV during the first month of therapy led us to suggest that the free virus production might consist of two phases: the first one is described by the term $1-\varepsilon$ used in the standard model, and the second by an exponential function described by the following equation: $\Psi(t) = (1-\varepsilon) \cdot [(1-\gamma) \cdot \Psi_0 \cdot e^{-\varphi \cdot t} + \gamma \cdot \Psi_0]$, where $1/\varphi$ represents the time constant of the second phase decline of virus production and $(1-\varepsilon)\gamma \cdot \Psi_0$ is the asymptotic value (Ψ_{asym}) of the free virion production rate.

We then applied this multiphase model to 72 HBeAg-negative patients treated with LMV monotherapy and with Peg-IFN2a or with the two drugs combined in an international phase Ⅲ study[25]. Applying the new model, we obtained a better fitting of the serum HBV-DNA multiphasic decline, as compared to the standard biphasic model (Figure 4), in 95% of the patients, and we could identify one additional phase of decline of viral production between days 4 and 14 in all LMV-treated patients.

We can hypothesize that two main biological mechanisms form the basis of this phase: a further reduction of virus production rate combined with an initial reduction in the number of infected cells. The additional antiviral effect of LMV inhibiting HBV replication during the second phase could be explained in several ways. One reason could be the decline of the cccDNA or viral minichromosome, provided that LMV blocks its production, as some authors have hypothesized in an animal model of hepadna-virus infection[26].

However, the half-lives determined for duck hepatitis B virus[26] and woodchuck hepatitis virus[21] in animal models and for HBV DNA in a chimpanzee experimental infection[27] were much longer than that computed for the above patients. Whalley *et al*[28] have reported an average HBV-DNA half-life of 3.7 ± 1.2 d compared with the 2 d found in the second phase of HBV-DNA decline during spontaneous resolution in patients with acute hepatitis B. Another hypothesis is that the block of virus production caused by LMV may restore some specific cytotoxic lymphocyte immune-mediated responses[29], and may also activate antiviral effects *via* cytokines,

such as IFNγ and tumor necrosis factor α, which either reduce transcription or increase cccDNA degradation. It has been proven that such mechanisms inhibit HBV replication before hepatocellular damage in transgenic mice and chimpanzee acute hepatitis models[17,22].

However, further studies are needed to understand better the biological reasons for this viral load decline. It is interesting to notice how Peg-IFN2a-treated patients had declines in serum HBV DNA much different from those observed in LMV-treated patients[20], given that the classic biphasic pattern was present during the first month of Peg-IFN2a monotherapy in the majority of patients. In the group of patients treated with Peg-IFN2a, however, the first slope of viral load decline was much lower than in the LMV-treated group with a mean HBV-DNA half-life of 1.6 ± 1.1 d and 9.5 ± 3.0 h, respectively.

The slower HBV-DNA decline during the first phase in patients treated with Peg-IFN2a monotherapy may have been caused by a decrease in virus production, similar to that induced by LMV with different kinetics, or to increased free virus clearance. Under the latter assumption, we noticed that the simulation of viral load decline did not fit with the experimental data, therefore, it appears more reasonable to accept the theory that Peg-IFN2a inhibits HBV replication inside the infected cells at a slower rate than LMV does.

The overall extent of inhibition of viral production induced by Peg-IFN2a was slightly higher (88%) than that reported by Sypsa *et al*[19] for Peg-IFN2b (82.6%). Such a difference emerged using our model, which allowed us to compute and study the antiviral effects of Peg-IFN during its whole period of action and not only during the first 4 d, as in the model of Sypsa *et al*[19].

Consistently, we could also prove that the extent of inhibition of viral production, in patients treated with LMV and Peg-IFN2a combination therapy, was comparable to that with LMV monotherapy, but the viral load decline was faster in the first phase (1/HBV-DNA decay constant: 2.79/d *vs* 1.91/d in LMV, $P < 0.005$) than in the second phase (1/HBV-DNA decay constant: 0.44 *vs* 0.34, $P = 0.023$).

In agreement with the fact that the molecular targets of LMV and IFN are different, these findings suggest that the mechanisms of IFN to inhibit HBV replication could be complementary and additive to those of LMV.

The experimental data reported in our study[20] proved also that the serum HBV-DNA decline after 4-5 wk of antiviral therapy tends to flatten in a large group of patients regardless of the treatment arm. If we assume that the viral load decline observed from day 14 onward depends uniquely on infected cell clearance, we can explain the slow decline in HBV-DNA levels after the first month of therapy, only accepting the reduction in the initial clearance rate of infected cells.

In the past, many efforts have been made to model variations in the immune response during viral infections[30], but they have led to a very complex system of differential equations without any effective result. Thus, with our model, we simply hypothesized a negative feedback on the immune clearance activity caused by a reduction in the number of infected cells, which is one major factor that induces cytotoxic immune responses.

The strength of the negative feedback, as modeled by the parameter "k", was computed by fitting the HBV-DNA serum decline, which is usually detectable up to the third month of therapy. This allowed a more realistic simulation of the decline in HBV DNA and infected cells during the remaining period of therapy. It is noteworthy that, at the end of therapy, the mean logarithmic reduction in the HBV-infected hepatocyte fraction, computed by our model, was comparable in patients treated with Peg-IFN2a and LMV monotherapy (-3.30 log10 *vs* -3.31 log10, $P > 0.05$), but significantly higher in patients treated with combined Peg-IFN2a + LMV as compared to LMV monotherapy (-5.02 log10 *vs* -3.31 log10, $P = 0.028$).

These findings suggest that Peg-IFN2a, in spite of a lower inhibition of HBV replication, has an equivalent, if not higher, impact on infected cell clearance than that of viral polymerase inhibitors. This could be the direct consequence of PEG-IFN2a immunomodulatory activity that polymerase inhibitors are lacking.

CONCLUSIONS AND PERSPECTIVES

Using our new multiphasic model, we could describe for the first time the second phase decrease in viral production that occurs mainly between days 4 and 14 in patients receiving LMV, alone or in combination.

Using the new model, we could prove that both IFN and LMV have inhibitory activities on HBV replication, but different kinetics. Their different mechanisms of action appear to work in synergy and cause faster declines in viral production during the first 2 wk of therapy in patients treated with both drugs.

The new model successfully describes the HBV infection dynamics far beyond the third month of antiviral therapy, under the assumption that the sum of infected and non-infected cells in HBeAg-negative chronic hepatitis B patients remains roughly constant during therapy, and both target and infected cells concur in hepatocyte turnover. These findings suggest that the ongoing production of new HBV-infected cells may occur after the division of already infected cells *via* transmission of the mini-chromosome-like cccDNA, when their immune-mediated clearance is reduced during long-term therapy. This might explain experimental data that report the persistence of high cccDNA levels even after 1 year of treatment with adefovir[31].

In the future, it will be very important to develop new models for a better simulation of HBV dynamics during various antiviral treatments, in order to predict the outcome of the infection after treatment discontinuation in every single patient, as is already available for hepatitis C patients[32]. The introduction of additional parameters that describe the HBV intrahepatic condition, related to infected hepatocytes and cccDNA copies, together with immune response markers in terms of immunoactivation and immunocompetence, could improve significantly the comprehension of the various profiles of response during

and after antiviral treatment. In clinical practice, these new models could allow the development of simulators of treatment response that will be used as an "automatic pilot" for tailoring antiviral therapy in chronic hepatitis B, as well as chronic hepatitis C patients.

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TOPIC HIGHLIGHT

Alberto Pilotto, MD, *Series Editor*

Hepcidin modulation in human diseases: From research to clinic

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Abstract

By modulating hepcidin production, an organism controls intestinal iron absorption, iron uptake and mobilization from stores to meet body iron need. In recent years there has been important advancement in our knowledge of hepcidin regulation that also has implications for understanding the physiopathology of some human disorders. Since the discovery of hepcidin and the demonstration of its pivotal role in iron homeostasis, there has been a substantial interest in developing a reliable assay of the hormone in biological fluids. Measurement of hepcidin in biological fluids can improve our understanding of iron diseases and be a useful tool for diagnosis and clinical management of these disorders. We reviewed the literature and our own research on hepcidin to give an updated status of the situation in this rapidly evolving field.

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Key words: Hepcidin; Iron homeostasis; Erythropoiesis; Hemochromatosis; Anemia; Liver diseases

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INTRODUCTION

Hepcidin is a circulating peptide hormone that regulates the entry of iron into plasma. It is primarily, but not exclusively, secreted by hepatocytes and is highly conserved among different species. The mature bioactive form of hepcidin is a peptide of 25 amino acids that derives from a precursor (pre-prohepcidin) of 84 amino acids that undergoes two enzymatic cleavages. Other isoforms of 20 and 22 amino acids are detectable in human serum and urine, although the biological significance of these isoforms is uncertain[1-3]. There is evidence that hepcidin is also expressed in the heart, kidney, adipose tissue, pancreas and haematopoietic cells, although the biological relevance of extra-hepatic hepcidin is not well defined yet (see below).

Targeted deletion of the *HAMP* gene in mice or mutations in the human *HAMP* gene result in the most severe forms of iron-overload disease. Conversely, increased expression of hepcidin leads to decreased iron absorption and iron deficient anaemia. Hepcidin, therefore, is a negative regulator of iron transport into plasma. It acts by binding to ferroportin, the only known cellular iron exporter, causing ferroportin to be phosphorylated, internalized, ubiquitylated, sorted through the multivesicular body pathway and degraded in lysosomes[4,5]. The interaction between hepcidin and ferroportin can explain the systemic regulation of iron metabolism. In fact, hepcidin is mainly targeted to duodenal enterocytes, where it regulates dietary iron absorption, and to macrophages, where it inhibits the

release of iron derived from senescent erythrocytes. By modulating hepcidin production, an organism controls intestinal iron absorption, iron uptake and mobilization from stores to meet body iron need[6,7].

REGULATION OF HEPATIC HEPCIDIN PRODUCTION: *IN VITRO* STUDIES AND ANIMAL MODELS

Hepcidin is modulated by different stimuli, which act as positive or negative regulators. There are four main active regulation pathways (erythroid, iron store, inflammatory and hypoxia-mediated regulation) that control hepcidin production through different signalling pathways (Table 1). These pathways must be closely coordinated to match iron supply to erythropoietic demand and, in turn, to maintain adequate plasma iron concentrations.

Positive regulation of hepcidin transcription

Under normal conditions, iron store and inflammatory regulation activate hepcidin transcription in the hepatocytes through the bone morphogenetic proteins (BMPs)/SMAD4 and signal transducer and activator of transcription-3 (STAT-3) pathways, respectively[6]. Binding of specific BMPs by hepatocyte BMP receptors results in phosphorylation of receptor SMADs (R-SMADs) within the cytosol. These phosphorylated R-SMADs complex with SMAD4, and the complex of phosphorylated R-SMAD and SMAD4 then activates transcription of *HAMP*[8]. Accordingly, *in vivo* and *in vitro* experimental studies showed that specific BMPs can activate *HAMP* transcription[9] and a liver-specific *Smad4*-knockout (KO) abrogates hepcidin gene transcription in mice leading to massive iron overload[10]. The hemochromatosis protein HFE, transferrin receptor 2 (TfR2) and the membrane isoform of hemojuvelin (mHJV) are all positive modulators of *HAMP* transcription and, when defective, lead to hemochromatosis (HH) in humans and HH-like phenotype in knock-out animal models[7].

HFE: Recent studies *in vitro* and in animal models clarified the crucial role of HFE as a hepatocyte iron sensor and upstream regulator of hepcidin[11,12]. Several mechanisms have been proposed by which HFE regulates iron metabolism. HFE competes with transferrin for binding to transferrin receptor (TfR)-1, lowering iron uptake into cells[13,14]. Alternately, there is more recent evidence supporting a role for HFE as an important component of a larger iron-sensing complex that involves interactions with diferric transferrin, TfR1 and TfR2 at the plasma membrane of hepatocytes[15-17]. Defective HFE protein prevents formation of a functional iron sensor and signal transduction effector complex leading to dysregulated hepcidin expression as observed in human hereditary hemochromatosis[18-20] and mouse models of this disease[21]. Recent findings also support other HFE-dependent mechanisms of regulation of iron homeostasis through post-transcriptional regulation of Zip14, a metal transporter that mediates non-transferrin-bound iron into cells[22].

TfR2: Homozygous mutations of *TfR2* have been linked to type 3 HH[23]. Animal models of mutated or knock-down *TfR2* confirmed human findings indicating that TfR2 plays an important role in iron metabolism[24-26]. It has been shown that diferric transferrin levels may increase the stability of TfR2 protein and it has been postulated that TfR2 is a sensor of serum iron levels and works to modulate iron absorption through the induction of the iron regulatory hormone hepcidin[27,28]. Accordingly, mutated or absent TfR2 is associated with reduced hepcidin expression either in animal models or in humans[18,24]. TfR2-induced regulation of hepcidin synthesis likely occurs through interaction with HFE to form an iron-sensing complex that modulates hepcidin transcription through the activation of a still unclear signalling pathway upstream or independent of SMAD4[7,29]. Interestingly, hepatic *HJV* mRNA level was lower in complete TfR2 knock-out[25] than in controls suggesting an interaction between TfR2 and HJV in the regulation of hepcidin expression. This finding has been recently confirmed to also occur in humans (Pelucchi *et al*, Haematologica in press).

HJV: As BMP co-receptor, HJV is strongly involved in the regulation of hepcidin transcription through the BMP/SMAD4 pathway[9,30,31]. Accordingly, *Hjv*-mutant mice exhibit iron overload as well as a dramatic decrease in hepcidin expression[32]. HJV is synthesized as a membrane-GPI-linked protein by hepatocytes but is also abundant in muscle. HJV exists in 2 forms: a membrane-bound form (m-HJV) and a soluble one (s-HJV), which *in vitro* reciprocally regulate hepcidin expression in response to opposite iron changes[33].

IL-6 and cytokines: Under inflammatory conditions, IL-6 and other cytokines induce *HAMP* transcription by activating STAT3 signalling to the hepcidin gene promoter[2,34,35]. The inflammatory regulatory pathway is thought to have a dominant effect on the other regulatory pathways as shown by the fact that inactivation of HFE does not affect the increase of hepcidin expression and the hypoferremia induced by inflammation. However, STAT3 activation requires the presence of SMAD4 because absence of SMAD4 prevents STAT3-mediated hepcidin gene expression[10].

Negative regulation of hepcidin transcription

Hypoxia, anemia, increased erythropoiesis and reduced iron stores all negatively regulate hepcidin expression. Hypoxia and anemia regulate the production of erythrocytes through synthesis of erythropoietin (EPO) and are the two main signals that increase iron absorption independently of iron stores to meet the need of iron for erythrocyte production[36,37].

Anemia could mediate hepcidin suppression

Table 1 Positive and negative regulators of hepcidin production, signalling pathways involved and human diseases either caused by defective function of the regulatory protein (Hemochromatosis, IRIDA) or inducing activation or suppression of the regulatory pathway

Positive regulators	Signalling pathway	Human disease	Negative regulators	Signalling pathway	Human disease
Inflammation [IL-6, IL-1α]	STAT-3	Anemia of chronic disease	Hypoxia [HIF1, sHJV, ROS]	HIF-1, BMPs/SMAD Oxygenases inhibition	--
Iron stores [mHJV]	BMPs/ SMAD4	Hemochromatosis	Iron stores [Matriptase2, s-HJV, GDF15]	BMPs/SMAD inhibition	IRIDA, iron deficiency
Iron stores [HFE, TfR2]	BMPs/ SMAD4	Hemochromatosis	Erythropoiesis [GDF15, others?]	BMPs/SMAD inhibition others?	Thalassemia, CDA1
Liver metabolic activities	C/EBPα		Erythropoietin?	C/EBPα block	
Oxygenases	?		Oxidative stress [ROS]	C/EBPα, STAT3 block; Oxygenases inhibition HDAC activation	Hepatitis C viral and alcoholic liver disease

IRIDA: Iron refractory iron deficiency anemia; CDA1: Congenital dyserythropoietic anemia type 1; Oxygenases: Family of 2-oxoglutarate-dependent oxygenases including prolyl and aspariginyl hydroxilases, PHDs and FIH. References: Andrews[6], De Domenico *et al*[7], Braliou et al[42], Choi *et al*[43], Nemeth[38] , Pinto *et al*[39], Pak *et al*[40], Tanno *et al*[41], Lakhal *et al*[54], Tamary *et al*[55], Silvestri *et al*[57], Silvestri *et al*[59], Miura *et al*[101], Harrison-Findik[46].

through multiple mechanisms including increased EPO or erythropoietic activity, increased iron demand or liver hypoxia[38]. Pinto *et al*[39] found that EPO at high concentration is able to inhibit hepcidin expression *in vitro*, *via* signalling through EPO receptor and C/EBPα regulation. This finding contrasts with that previously reported by Pak *et al*[40] who showed that hepcidin regulation depended on erythropoietic activity and was not directly mediated by anemia, tissue hypoxia, or EPO in murine models and that secondary changes in plasma and tissue iron would also be expected to contribute to hepcidin regulation. The nature of the erythropoietic regulator of hepcidin is still uncharacterised, but may include one or more proteins released during active erythropoiesis. Recent observations in thalassemia patients has suggested that one of these regulators could be the cytokine growth differentiation factor-15 (GDF15)[41].

Hepcidin is suppressed in human cultured hepatoma cells exposed to hypoxia[42] but the physiological relevance and the mechanisms of hepcidin regulation by hypoxia are still uncertain and conflicting. Hypoxia-inducible factor (HIF)-1 and reactive oxygen species (ROS) have been both implicated in hepcidin regulation either directly or through modulation of 2-oxoglutarate-dependent oxygenases, respectively[42-44].

Studies *in vitro* and *in vivo* in animals[45,46] have recently shown an alcohol-dependent decrease of hepcidin expression possibly through ROS-induced down-regulation of C/EBPα. Two other studies analysed the effect of 7 d alcohol exposure in HFE KO mice and results were discrepant, one showing an additive effect of alcohol on hepcidin down-regulation[47] and the other not[48]. There remains however a major discrepancy between these experimental findings and the absence of increased iron deposits in alcohol-fed animal models[45,49], and clinical data in humans indicate a generally mild effect of chronic alcohol consumption on iron stores even in patients with hemochromatosis[50-53].

GDF15: GDF15 is a divergent member of the transforming growth factor-β superfamily that is secreted by erythroid precursors and other tissues. It has been identified as an oxygen-regulated transcript responding to hypoxia and as a molecule involved in hepcidin regulation[41,54]. This is intriguing considering the strong interaction between iron and oxygen and indicates that some homeostatic systems for iron and oxygen are responsive to both stimuli. Tanno *et al*[41] showed that serum from thalassemia patients suppressed hepcidin mRNA expression in primary human hepatocytes and depletion of GDF15 reversed the hepcidin suppression. They suggested that GDF15 overexpression arising from an expanded erythroid compartment contributed iron overload in thalassemia syndromes by inhibiting hepcidin expression, possibly by antagonizing the BMP pathway. Very high levels of serum GDF15 were also observed in patients with congenital dyserythropoietic anemia type 1 (CDA I) suggesting that GDF15 contributes to the inappropriate suppression of hepcidin with subsequent secondary hemochromatosis in these patients[55]. Very recently Lakhal *et al*[54] partially elucidated some of the mechanisms regulating GDF15 and demonstrated robust and sensitive up-regulation of GDF15 mRNA and secreted protein in response to iron depletion in a range of human cell lines and *in vivo* in humans. They also demonstrated that this up-regulation was independent of HIF providing support for the involvement of a novel iron and oxygen-sensing pathway. Whether regulation of GDF15 by intracellular iron provides a mechanism by which intracellular iron might directly influence hepcidin production is unclear and requires further analysis.

HIF, ROS and Oxygenases: Hypoxia is primarily sensed in vertebrates by the HIF family of transcription factors. Under normal conditions, hydroxylation of HIF results in its recognition by the von-Hippel–Lindau (VHL) ubiquitin ligase, which targets it for degradation. In the absence of oxygen, HIF proteins

are stabilized and function as transcription factors[37]. Mice with a liver-specific deletion of HIF1α, even if they are maintained on a low iron diet, show increased hepcidin expression because they have an impaired hypoxic response and cannot normally down-regulate hepcidin gene transcription[44]. In contrast, mice with a liver-specific deletion of the *Vhl* gene show extremely low levels of hepcidin expression[44]. The same authors showed that HIF1α can bind to the mouse and human *HAMP* promoter suggesting that HIF-family members can directly and negatively regulate hepcidin expression[6,44]. Thus, the VHL/HIF pathway could be an essential link between iron and oxygen homeostasis and hepcidin regulation *in vivo* that through coordinated down-regulation of hepcidin and up-regulation of erythropoietin and ferroportin, mobilizes iron to support erythrocyte production. Conflicting data were, however, obtained in hepatoma cell lines in which over-expression, knockout and chromatin immunoprecipitation (Chip) assays failed to demonstrate the involvement of HIF-1 in the regulation of hepcidin promoter[42,43]. Choi *et al*[43] found that hypoxia-induced hepcidin suppression was related to ROS levels which prevented the binding of transcription factors, CCAAT/enhancer-binding protein alpha (C/EBPα) (a liver enriched transcription factor that accounts for developmental changes in liver metabolism after birth) and STAT-3, to the *HAMP* promoter. More recently, Braliou *et al*[42] showed that 2-oxoglutarate-dependent oxygenases (which include prolyl and asparagine hydroxylases, PHDs and FIH) were important to maintain high hepcidin mRNA expression in a HIF-1-independent manner These enzymes depend on molecular oxygen, 2-oxoglutarate and Fe^{2+} and act on their main substrate HIF-1α causing its inactivation and degradation by protein hydroxylation. Hypoxia and generation of ROS inhibit protein hydroxylation and cause stabilization and activation of HIF-1α[37]. Braliou *et al*[42] demonstrated that hypoxia and chemical agents inhibiting the 2-oxoglutarate-dependent oxygenases repress hepcidin expression in hepatoma cell lines opening the way for further experimental studies to identify downstream factors mediating hepcidin regulation.

Soluble HJV: It has been shown that s-HJV can bind BMP and function as a competitive antagonist of m-HJV, leading to decreased hepcidin expression[33] and that chronic s-HJV injection in mice causes iron overload[56]. Silvestri *et al*[57] demonstrated that s-HJV is the product of a furin cleavage at the C-terminus of the protein that occurs mainly in the endoplasmic reticulum and that it is up-regulated in conditions of iron deficiency and hypoxia. They suggested that hypoxia and iron deficiency activate furin to release s-HJV and to rapidly reduce the amount of m-HJV, inhibiting hepcidin production[33,57]. These events may occur in the same cells (hepatocytes) that produce hepcidin in order to suppress hepcidin up-regulation by an autocrine mechanism.

Transmembrane serine protease 6: Recently, Du *et al*[58] described *mask*, a recessive, chemically induced mutant mouse phenotype, characterized by progressive loss of body, but not facial, hair and microcytic anemia. The *mask* phenotype results from reduced absorption of dietary iron caused by high levels of hepcidin. High *Hamp* mRNA levels were in fact observed in the liver of *mask* homozygotes despite anemia, a finding consistent with insensitivity to low iron stores and failure to suppress hepcidin synthesis. A splicing defect was found in the transmembrane serine protease 6 gene *Tmprss6* in the *mask* mutant. This gene is expressed in a limited number of tissues, but the major site of expression is the liver in both mice and humans. Du *et al*[58] demonstrated that TMPRSS6 cotransfection strongly inhibited *Hamp* reporter gene activation by each stimulus (IL-1α, IL-6, BMP2-4-9) in HepG2 cells, whereas the *mask* mutant version of TMPRSS6 showed a modest inhibitory effect. This indicates that TMPRSS6-dependent pathway predominates over all known *Hamp*-activating pathways and that TMPRSS6-mediated *Hamp* suppression is determinant for acquiring adequate iron uptake from dietary sources. They hypothesized that TMPRSS6, also known as matriptase-2, participates in a transmembrane signalling pathway triggered by iron deficiency and independent to the known *Hamp* activation pathways and demonstrated that the proteolytic activity of matriptase-2 is determinant to hepcidin suppression activity. Recent experiments provided evidence that this serine protease inhibits hepcidin by cleaving HJV from the plasma membrane and has no cleavage activity on s-HJV[59]. The tissue-restricted, strong liver expression of matriptase-2 is logical if we consider that its activity responds to iron deficiency, in order to suppress the mHJV-BMPs pathway of hepcidin activation. The authors suggest that the pathway may still be modulated by s-HJV, further increasing the inhibition of hepcidin transcription induced by matriptase-2 alone.

HEPCIDIN MEASUREMENT IN HUMAN DISORDERS: PAST, PRESENT AND FUTURE

Since the discovery of hepcidin and the demonstration of its pivotal role in iron homeostasis, there has been a substantial interest in developing a reliable assay of the hormone in biological fluids. Measurement of hepcidin in biological fluids can improve our understanding of iron diseases and be a useful tool for diagnosis and clinical management of these disorders. However, this has proven to be a very challenging task.

Hepcidin assay in biological fluids

Inherent problems: The development of traditional immunochemical methods based on the production of specific anti-hepcidin antibodies has been hampered by several factors, including: (1) the small size (25 amino acids) and the compact structure of the peptide,

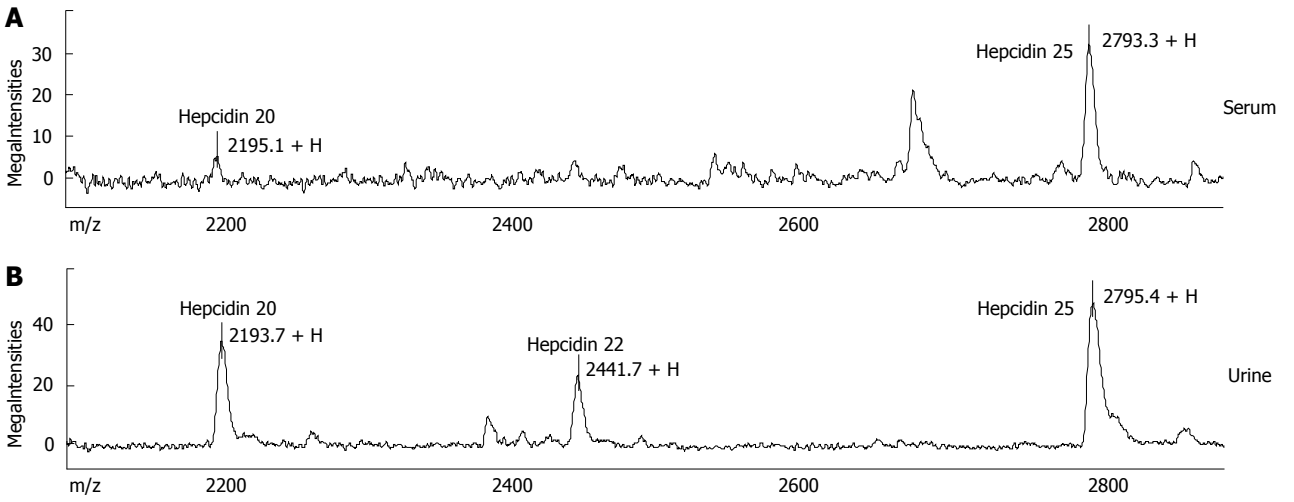

Figure 1 Typical mass spectra of human serum (A) and urine (B) samples by SELDI-TOF-MS in a control subject. In addition to hepcidin-25, two isoforms truncated at the N-terminus are detectable. The 20 aa isoform is detectable in both urine and serum, while the 22 aa isoform is found only in urine.

with few antigenic epitopes; (2) the high degree of conservation between animal species[60], with ensuing difficulties in elicitation of an appropriate immune response in host animals; (3) the limited availability of the antigen. Indeed, the production of synthetic hepcidin in its hairpin native conformation determined by four disulfide bonds among the eight cysteine residues (near one fourth of the molecular weight)[61] is a complex procedure, as well as the isolation of hepcidin from urine[62]. A further problem is represented by hepcidin tendency to aggregate and to stick to laboratory plastic tubes, necessitating the need for careful handling and for standardized pre-analytical procedures.

"First generation" methods: Early after hepcidin discovery, an ELISA kit able to detect serum pro-hepcidin became commercially available[63], based on an antibody recognizing an epitope outside the 25 amino acid sequence of the bioactive peptide. With few exceptions, this assay generally failed to give clinically useful information, since no correlation was reported with iron status and/or absorption[64,65]. Most of the human studies published so far have relied on an immunodot assay for urinary hepcidin based on selective extraction of the peptide from urine by cation-exchange chromatography and its subsequent quantification by chemiluminescence using rabbit anti-human hepcidin primary antibodies[66]. Although this method provided very useful information on hepcidin regulation in human diseases[18-20,67,68], it was quite laborious, and suitable only for relatively small series of patients. To circumvent inherent limitations of immunochemical methods, several research groups have focused on novel technologies, particularly on Mass Spectrometry (MS)-based methods, that rely on direct determination of the molecule of interest without the need for specific antibodies. Among these techniques, Surface-Enhanced Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (SELDI-TOF-MS) has emerged as a proteomic technique particularly promising for rapid direct detection of small sized biomarkers[69], like hepcidin. SELDI-TOF-MS combines matrix assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to surface chromatography. Depending on their chemical characteristics, peptides/proteins of interest are captured by specific interactions with protein surfaces ("chip-arrays") that are used as a laser desorption ionization target. After the enriching step, proteins that do not bind to the surface are removed by a simple wash step, while bound proteins are analyzed by MS. Good preliminary results in hepcidin measurement were reported by several groups[70-73]. In particular, the SELDI-TOF-MS assay was able to discriminate different clinical conditions on the basis of the expected variations in hepcidin levels[70,72], and correlated well with the immunodot assay in head-to-head comparison[70]. An important advantage of the SELDI-TOF-MS technique is the potential to evaluate not only the predominant 25 amino acid form, but also the forms with two peptides shorter at the amino terminus, i.e. hepcidin-22 and hepcidin-20 (Figure 1; also see below). Nevertheless, an inherent limitation of first generation SELDI-TOF-MS hepcidin assays (and in general, of all MS-based methods) is that peak heights in mass spectra do not always reflect absolute concentrations in clinical samples, because of competition during binding steps, and variations in ionisation efficiency. This implies that results can be expressed only in semi-quantitative arbitrary units, unless the use of a proper internal standard is implemented. But this again is a difficult task. Ideally, an internal standard is represented by a synthetic hepcidin-related peptide with no overlap with the endogenous peptide or other common peaks in serum/urine mass spectra, so that it could be spiked at known concentration to the clinical sample and co-analysed. Quantification of endogenous peptide is then obtained through the ratio internal standard: endogenous peptide. Murphy *et al*[74] first described a MS-based quantitative assay for either human or mouse

Table 2 Recently published methods for hepcidin quantification in biological fluids

Method	Reported normal range in serum (No. of controls)	Intra-assay precision (CV[1]) (%)	Internal standard	Note	LLOD[2]	Reference
C-ELISA	29-254 ng/mL (males) 17-286 ng/mL (females) (*n* = 114)	5-19	n.a.	Also used to quantify hepcidin in urine	5 ng/mL	Ganz[76], 2008
Quantitative SELDI-TOF-MS	9-45 ng/mL (males) 7-15 ng/mL (females) (*n* = 23)	5.7-11.7	Des-Asp Hepcidin-24 (deletion of aspartate residue at position 25)	Also used to quantify hepcidin in urine	3 ng/mL	Swinkels[77], 2008
Quantitative SELDI-TOF-MS	50 ng/mL (average in females) (*n* = 24)	8-9	Stable isotope labelled hepcidin ($^{13}C/^{15}N$ phenylalanine at position 9)		10 ng/mL	Ward[79], 2008
Micro-HPLC tandem tandem MS	1-19.5 ng/mL (males) 1-6.5 ng/mL (females) (*n* = 10)	4.8-21	Stable isotope labelled hepcidin ($^{13}C/^{15}N$ isoleucines at position 6-8)		1 ng/mL	Kobold[78], 2008
Liquid Cromatography tandem MS	n.a.	8.7-12.5	Stable isotope labelled hepcidin ($^{13}C/^{15}N$ glycines at position 12-20)	Only used in urine for the moment	n.a.	Bansal[75], 2008
Hepcidin binding domain based test	39-88 ng/mL (*n* = 40)	< 5	n.a.	Potential for measuring true biologically active hepcidin (e.g. able to bind ferroportin)	n.r.	De Domenico[1], 2008

n.a.: Not applicable; n.r.: Not reported; [1]Coefficient of variation. CV ranges refer to samples with different hepcidin concentrations. With all methods, variations in repeated assays were higher at the lower hepcidin concentration; [2]Lower limit of detection.

hepcidin in serum using liquid chromatography tandem MS (LC-MS/MS) and calcitonin gene-related peptide (CGRP) as the internal standard. While a good accuracy was reported, the suitability of CGRP as non-hepcidin internal standard cannot be considered ideal because of its differences from the endogenous peptide in terms of mass, hydrophobicity, p*I*, and net charge[75].

Recent advances by "second generation" assays: This year has witnessed substantial advances in quantitative methods for hepcidin measurement in serum and/or urine. A variety of different approaches have been used by several research groups, summarized in Table 2. One of the most innovative was described by De Domenico *et al*[1]. They first identified the hepcidin binding domain (HBD) on the ferroportin molecule, and then synthesized a 19 amino acid corresponding peptide (spanning from amino acids 324 through 343 of the fourth extracellular loop of ferroportin). This peptide was then used in a competitive assay, i.e. by determining the ability of serum samples to compete with radioactive hepcidin for binding to the HBD peptide. In mice with targeted deletions of either *HAMP* or *HFE* gene, serum hepcidin measured by this method was found to vary as expected, as it did in a small group of healthy volunteers. This assay looks promising as an effective measure of true biologically active hepcidin, but needs further validation.

Ganz *et al*[76] were successful in obtaining sufficient amount of anti-hepcidin antibody to develop the first competitive enzyme-linked immunoassay (C-ELISA) for human hepcidin in a simple format (96-well plates) applicable to a relatively large series of patients. On the other hand, Swinkels *et al*[77] reported good quantification of hepcidin in both serum and urine updating the SELDI-TOF-MS protocol with the use of a proper internal standard clearly distinguishable from the endogenous peptide, i.e. a synthetic hepcidin-24 lacking the amino-terminal asparagine residue. Moreover, other groups working on MS-based methods reported reliable hepcidin quantification using stable isotope labelled 25 amino acid peptides with masses different from endogenous hepcidin because of the introduction of $^{13}C/^{15}N$ at different amino acid positions[75,78,79]. Interestingly, the C-ELISA and the updated SELDI-TOF-MS protocol were used to measure hepcidin in series of serum/urine pair samples from healthy donors[76,77], yielding quite similar results in terms of fractional excretion of the peptide (3%-5%). A preliminary direct head-to-head comparison of these two methods in split serum samples from the same individuals showed a high degree of correlation (Spearman's rho up to 0.94; (Girelli D, unpublished observation)). Moreover, similar comparative analyses in rigorously selected healthy subjects without any potential confounder of iron status (e.g. subclinical inflammation or iron depletion) showed that the two methods were almost equally effective in reflecting the expected subtle physiologic variations of serum hepcidin as a function of established indices of body iron stores, with Spearman's rho ranging from 0.7 to 0.8 (Girelli D, unpublished observation). In perspective, the two methods may be of complementary implementation in clinical and/or research setting. SELDI-TOF-MS has the disadvantage of relying on relatively expensive equipment and the need of dedicated skilled personnel. On the other

hand, it has the positive advantage of being able to detect the two isoforms truncated at the N-terminus, i.e. hepcidin-22 and hepcidin-20. At present, the actual biological significance of these post-translational modifications is uncertain. Studies by Nemeth *et al*[3] have demonstrated that the five N-terminal amino acids are essential for the interaction with ferroportin, and thus for modulation of iron homeostasis. On the other hand, the 20 amino acid isoform has been demonstrated to possess greater antimicrobial activity than hepcidin-25[1,62]. Thus, the smaller forms may not simply reflect the catabolism of the "iron active" hepcidin-25 as postulated by some authors[80], but rather the generation of more potent defensins with antimicrobial peptides[1]. Further studies are needed in this direction, as it appears that we are just starting to know the complex role of hepcidin at the crossroads between iron homeostasis and infectious/inflammatory conditions.

In contrast, the C-ELISA method has the potential for a more widespread diffusion into clinical/hospital settings, since it does not need any specific equipment. However, the specificity of the antibody used with respect to various hepcidin isoforms remains to be verified.

Hepcidin in human diseases

Until recently, most of the studies of hepcidin in humans have relied on a human urinary hepcidin assay and on comparisons between patients and small groups of normal subjects without distinction between gender and age, all factors that might influence hepcidin concentrations. Very recently, Ganz *et al*[76] reported data on 114 healthy subjects, showing that the medians of hepcidin concentrations, measured by C-ELISA, significantly differed between men and women (112 and 65 ng/mL, respectively), likely due to difference in iron stores. They also showed a trend for age-related increase in serum hepcidin in both genders needing further confirm, and that there was a direct correlation between serum hepcidin and ferritin concentrations within the range of normality for ferritin.

Iron overload: In most cases iron overload results from inadequate hepcidin production relative to body iron stores[6,7,81]. However, the causes that lead to reduced hepcidin production are different and include: hereditary defects that disrupt one or another protein involved in the normal pathway of positive regulation of hepcidin transcription (HFE, TfR2, HJV) and hepcidin itself; hereditary defects of proteins involved in iron transport (hypo-transferrinemia); ineffective erythropoiesis leading to increased and endless iron need by the erythroid marrow independent of iron stores (iron loading anemias)[38]. Exceptions are the iron overload due to hereditary defects of ferroportin, in which urinary hepcidin was found to be variably increased[19,82,83] and pure transfusional iron overload in which the increased iron stores stimulate hepatic hepcidin production and can reach into the thousand of ng/mg creatinine[84]. In both these conditions, however, the cases tested are few and not homogeneous, and results need confirmation. There are no available data on aceruloplasminemia.

Hemochromatosis: Based on the current knowledge of the pathophysiological mechanisms of hepcidin regulation and on hepcidin measurement at either protein or mRNA level, hemochromatosis (HH) can be divided into two groups: primary iron overload disorders associated with defective or suppressed *HAMP* expression and ferroportin diseases.

The first group includes HH type 1, 2 and 3, which are determined by defects in four different genes (*HFE*, *TfR2*, *HJV* and *HAMP*). Since all these proteins are involved in the same pathway, the physiopathological mechanism leading to iron overload in all these forms of HH depends on absent or defective synthesis, or the inability to up-regulate hepcidin production appropriately in response to increased iron stores[6,7,11]. This in turn induces increased iron absorption in the plasma which exceeds the binding capacity of transferrin, causing the production of non-transferrin-bound iron and the accumulation of iron in parenchymal tissues. Thus, the differences among these forms of primary iron overload is quantitative (the amount of iron overload and the severity of iron-related damage), rather than qualitative (similar alterations of serum iron indices, similar iron distribution in the liver, same targets of iron deposition and damage). It can be hypothesised that the more important a defective protein is in the regulatory pathway of hepcidin transcription, the stronger is its suppression and the higher is the rate and amount of iron accumulation and, consequently, the severity of iron-related damage. In fact, in juvenile hemochomatosis (JH), hepcidin concentrations are lower than in adult forms of hemochromatosis, despite massive iron overload[19]. Figure 2 shows the concentration of urinary hepcidin measured by the same method in different forms of HH derived from personal and unpublished observations and on available data in the literature. For HFE-related HH patients we report only data collected at baseline. For the rare forms of HH, the available data on hepcidin are very limited in number and in most of these patients hepcidin measurement was not done at baseline. Thus, we took care, as far as possible, to select those still not fully iron depleted and sampled some time from phlebotomies, to limit the effect of phlebotomy-induced erythropoiesis and iron depletion on hepcidin synthesis.

Due to the higher prevalence of HFE-related HH, most of the data available on hepcidin measurements concern this form of HH. At diagnosis, when C282Y homozygous patients are iron overloaded, hepcidin values either at mRNA, urine or serum levels are only slightly lower than in controls[20,67,85-87]. Interestingly, in only one study hepcidin was measured also in the C282Y/H63D genotype, showing that it was higher

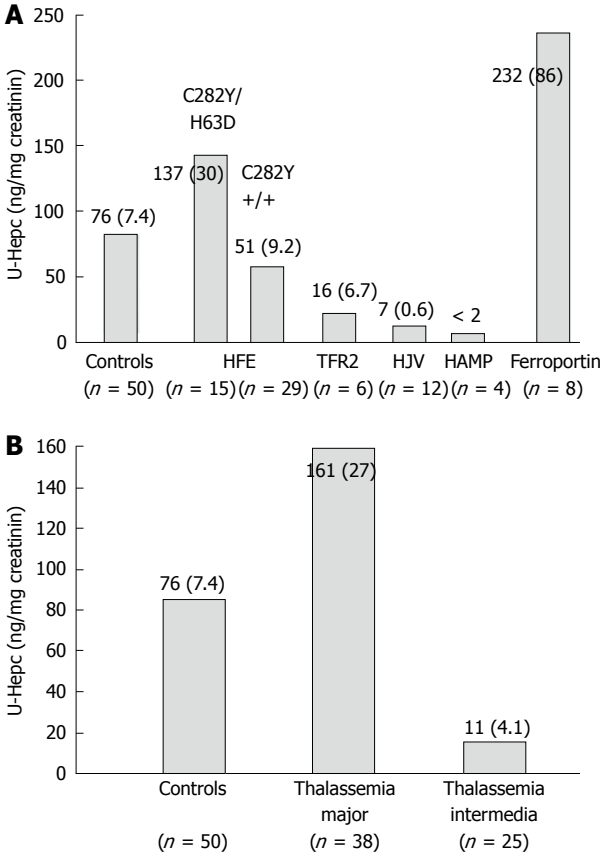

Figure 2 Mean (SE) values of urinary hepcidin (U-hepc), measured as described[20], in patients with different forms of hemochromatosis (A) and in patients with thalassemia major and intermedia (B) compared with healthy controls. Data collected from personal unpublished results and from Barisani *et al*[67], Cremonesi *et al*[82], Kattamis *et al*[90], Matthes *et al*[116], Nemeth *et al*[18], Origa *et al*[91], Papanikolau *et al*[117], Papanikolau *et al*[19], Piperno *et al*[20]. ($P < 0.05$: Controls *vs* C282Y +/+) ($P < 0.01$: Controls *vs* others; C282Y +/+ *vs* C282Y/H63D, HJV and Fp; HJV *vs* Fp; Thalassemia Major *vs* Intermedia). Statistical analysis was not performed for TFR2 and HAMP due to the small number of patients.

than in controls at diagnosis[20]. In both genotypes, however, the hepcidin/ferritin ratio (a derived parameter used to normalize hepcidin levels by the amount of iron overload) was significantly lower than in controls[20]. When analysed after iron depletion, patients of both genotypes, even at a time remote from the last phlebotomy, showed hepcidin concentrations significantly lower than that observed in iron overloaded patients and in controls. These findings allowed two considerations to be made: first, assuming that after normalization of iron stores HH patients return to their inborn status, van Dijk *et al*[87] concluded that hepcidin levels are innately low in HFE-HH; second, that patients with HFE-HH can modulate, although inappropriately, the chronic hepcidin increase in response to iron stores[20]. This impairment is less evident when the defect is mild as observed in patients with the C282Y/H63D genotype[20]. Based on the evidence that genetic background influences iron overload in HFE knockout mice and on recent findings in humans showing that some polymorphisms in the BMPs/SMAD4 signalling cascade can influence the iron phenotype in C282Y homozygotes[88], it can be hypothesised that phenotype variability in HFE-HH depends on the whole effect of major disease locus (HFE) together with genetic polymorphisms and modifiers on hepcidin production that can protect or aggravate the effect of the defective protein. Acquired factors may contribute to phenotype variability through modulation of hepcidin production in HFE-HH[89], but no data are available yet.

Recently, we demonstrated that in contrast to healthy subjects, hepcidin response after acute oral iron challenge was blunted in most patients with HFE-HH at diagnosis and after iron depletion[20]. This supports a role of HFE in the iron-sensing pathways regulating hepcidin synthesis. Interestingly, we also found a blunted response to acute oral iron challenge in a severe iron overloaded patient affected by type 3 HH due to a novel splicing defect of *TFR2* (Pelucchi *et al*, *Haematologica* in press). Although this single observation needs caution, it provides the first evidence *in vivo*, in humans, that TFR2 may act as a sensor of serum iron levels to modulate hepcidin production[27,28].

A handful of data is available for ferroportin disease (type 4 hemochromatosis) and these data are reported in Figure 2. The high concentration of urinary hepcidin has been ascribed to response to iron overload, but also to "hepcidin resistance" that may vary according to different mutations of ferroportin. However, there are not enough data to define how hepcidin is regulated in the two different forms of ferroportin disease (types A and B) and if hepcidin measurement can be of some help in distinguishing one type from the other.

Iron loading anemias: The erythroid regulator exerts a negative effect on hepcidin production that overwhelms the store regulator as shown by the marked iron overload which develops in hypotransferrinemia and iron loading anemias[38].

Thalassemia intermedia and thalassemia major are the best studied human models of hepcidin modulation by ineffective erythropoiesis alone and the combined and opposite effect of both ineffective erythropoiesis and transfusion dependent iron overload, respectively. Regular transfusions, in fact, induce a huge tissue iron accumulation, but also inhibit erythropoietic drive. As shown in Figure 2, hepcidin levels are markedly reduced in thalassemia intermedia, due to the erythropoietic drive and despite systemic tissue iron overload[90,91]. Similar results were observed in not transfused hereditary diserythropoietic and acquired sideroblastic anemias[19] that share common pathogenetic mechanisms of iron overload with thalassemia intermedia[36,38]. In thalassemia major, hepcidin production is higher than in thalassemia intermedia although still inappropriate to the massive transfusional iron loading that partially counteracts the erythropoietic-dependent hepcidin down-regulation[90-93]. However, there is a large variability in thalassemia major probably dependent on several factors that might influence hepcidin production: time of transfusion[93], iron chelation therapy, amount of iron overload.

Very few data are presently available for other iron loading anemias that share pathogenetic mechanisms of iron overload with thalassemic syndromes, such as sideroblastic and congenital dyserythropoietic anemias. As expected, hepcidin either at mRNA level in the liver or at protein level in the urine was found to be suppressed despite marked iron overload[19]. There are scanty and discrepant results in sickle cell disease where iron status and hepcidin regulation are influenced by various factors that may act in opposite directions: anemia and hypoxia that lead to suppression of hepcidin production, transfusional iron overload and inflammation that stimulate hepcidin synthesis by different pathways[93,94].

Due to the rarity of congenital hypo-transferrinemia in humans, most data on hepcidin regulation comes from studies in hypotransferrinemic mice which recapitulate the human disorder, showing very low hepatic mRNA levels that support the notion of a dominant erythroid signal in hepcidin regulation and the importance of low hepcidin levels in the development of iron overload[93-95]. There is a single study in a child with congenital hypo-transferrinemia which describes the modulation of urinary hepcidin during plasma transfusions[96]. The study suggests that hepcidin production is regulated by the balance between iron requirements of the erythroid marrow and iron supply by transferrin, in agreement with the concept that iron supply to the erythron is the most important factor influencing iron absorption and iron release from stores[36,96].

Transfusional iron overload: The majority of studies relate to thalassemic patients in which ineffective erythropoiesis and transfusions frequently cooperate to produce iron overload and induce opposite effects on hepcidin transcription as extensively described. In contrast, very few and sometimes contradictory data are available for patients with pure transfusional iron overload. Nemeth *et al*[84] showed that hepcidin can reach into the thousands of ng/mg creatinine in two polytransfused patients with myelodysplastic syndrome (MDS), suggesting that the ranges of variation for urinary hepcidin and serum ferritin induced by iron overload are actually very similar. A recent study in 20 patients with MDS and myelofibrosis reported different results, mostly suggesting suppression rather than induction of hepcidin synthesis[97]. However, the group was not homogeneous and included patients with different MDS subtypes and alterations of iron status such as iron deficiency, iron overload secondary to transfusions or ineffective erythropoiesis. Overall, these findings indicate that the causes of hepcidin down-regulation in patients with MDS and myelofibrosis are much more heterogeneous than in thalassemia and need to be defined in more selected series[98].

Other acquired iron overload disorders: Preliminary data are available for some common chronic liver diseases frequently associated with slight to moderate iron overload. Aoki *et al*[99] measured hepatic mRNA expression in patients with chronic hepatitis C showing that hepcidin expression did not correlate with markers of inflammation, but correlated with hepatic iron stores, suggesting that iron overload in chronic hepatitis C is not due to inappropriate hepcidin production. In contrast, Fujita *et al*[100] found that patients with chronic hepatitis C had serum hepcidin-to-ferritin ratios significantly lower than HCV negative controls and that this relative impairment of hepcidin production was fully reversible after successful HCV eradication by PEG-IFN plus ribavirin. The hypothesis that HCV might suppress hepcidin expression is also supported by recent experiments in HCV replicon cells and in HCV core-expressing Huh7 cells. Miura *et al*[101] found that hepcidin expression was inversely correlated with the amount of reactive oxygen species (ROS) production and that HCV-induced oxidative stress caused hypoacetylation of histones and inhibited binding of two positive regulators (C/EBPα and STAT3) of hepcidin transcription. Interestingly, anti-oxidants restored hepcidin expression in these cell lines and reduced HDAC activity in a dose-dependent manner. Hepatic hepcidin mRNA expression correlated with hepatic iron in advanced chronic liver disease and may also be affected by hepatic dysfunction[68]. A reduced hepcidin synthesis indeed might be one of the mechanisms leading to iron overload in advanced liver disease of any origin, but no further studies have clarified this issue.

Increased hepatic iron deposits have been frequently described in association with obesity and alterations of lipid or glucose metabolism, insulin resistance and non-alcoholic fatty liver (NAFLD) and variably named dysmetabolic or insulin-resistance hepatic iron overload syndrome (IR-HIO). Urinary hepcidin levels, although inappropriate for the iron overload, were indeed significantly higher in patients with dysmetabolic iron overload than in controls[67]. This finding has been also confirmed by others, as recently reviewed by Deugnier *et al*[89]. Hepcidin resistance or increased extra-hepatic production have been hypothesised[67,89] to explain these findings. Another explanation is that IR-HIO might be the result of an association of a mild-moderate, maybe polygenic, defect of hepcidin production and insulin resistance or metabolic syndrome (MS). These patients may retain some ability to increase hepcidin production in response to iron load as observed in subjects carrying the low expressing C282Y/H63D HFE genotype[20]. In IR-HIO patients, NAFLD and MS might induce hepcidin production through cytokine-mediated pathways[102] leading to the typical phenotype[103]. Further studies are needed to clarify this issue and the role of dysmetabolism in dysregulating iron regulatory pathways.

Iron deficiency: Urinary hepcidin levels were undetectable or low in patients with iron deficiency anemia[76,84], in agreement with the suppressive effect of deficient iron stores and iron deficient erythropoiesis

on hepcidin production. Very recently, the *TMPRSS6* gene has been identified as the hepcidin negative regulator required to sense low iron stores[104]. TMPRSS6 mutations cause the rare iron-refractory iron deficiency anemia (IRIDA)[105,106] and, in accordance with the suppressive effect of TMPRSS6 protein on hepcidin production, IRIDA patients show inappropriately elevated urinary hepcidin levels. This may explain the failure to absorb dietary iron despite systemic iron deficiency as well as the partial failure to respond to parenteral iron, which must be processed and then released by macrophages before being driven to the erythroid marrow[105,106].

EXTRA-HEPATIC PRODUCTION OF HEPCIDIN

Although the liver is the main site of hepcidin synthesis, recent studies demonstrated the presence of measurable amounts of hepcidin mRNA and protein in cells and tissues other than liver in humans and animals: heart, kidney, retina, monocytes and macrophages, splenocyte and alveolar cells, adipocytes and pancreatic β-cells[102,107-113]. In all these tissues the basal expression rate of hepcidin is lower than in the liver, suggesting a local role for hepcidin regulating iron homeostasis in these organs and tissues in an autocrine and paracrine fashion. On the other hand, it has been hypothesised that pancreatic hepcidin may contribute to the systemic hepcidin pool since it is exclusively synthesized by β-cells that secrete their product into the blood[108]. Nonetheless, this seems unlikely since expression of hepcidin in the pancreas is lower than in the liver and β-cells represent only a small portion of the total pancreatic parenchyma, in contrast to hepatocytes.

A major limitation of these studies is that they analysed hepcidin expression in response to only one of the major hepcidin regulators (iron, inflammation or hypoxia). In addition, only few evaluated the coupled modifications of hepcidin and ferroportin expression in response to such stimuli. There is emerging evidence, in fact, that hepcidin and its molecular target ferroportin may be expressed in the same cells, suggesting that ferroportin may be regulated by hepcidin generated within these cells independent of hepcidin in the circulation[107,108,113,114].

Most studies showed that hepcidin responds to an acute phase reaction caused by either LPS, turpentine, group A *Streptococcus* strain or *Pseudomonas aeruginosa*. They can induce a 20-80 fold increase of hepcidin expression in murine macrophages, splenocytes and retinal cells by a Toll-like receptor 4 (TLR-4) dependent pathway[107,110,112], and in human monocytes *via* IL-6 induction, which is TLR-4 independent and involves STAT-3 dependent activation[114]. It is suggested that formation of hepcidin may locally contribute to the development of iron retention as part of the innate defensive mechanism generally aimed at reducing the availability of the essential nutrient iron from pathogens. This may be important for tissues such as the retina and the brain, which are protected by blood barriers or at inflammatory sites with poor perfusion where circulating hepcidin is scanty. In particular, it has been proposed that hepcidin formation by activated monocytes/macrophages may result in biologically significant accumulation of this peptide in the inflammatory environment[114]. Hepcidin produced by monocytes targets membrane bound ferroportin primarily as a secreted peptide in an autocrine way, but hepcidin also affects, although to a much lesser extent, ferroportin expression within the cell, suggesting two pathways of hepcidin trafficking within macrophages.

Further studies are needed to understand the function of hepcidin in extrahepatic tissues and to evaluate possible influences at the systemic level. In fact, it has been hypothesised that in massively obese patients hepcidin production by adipocytes might contribute to the development of iron deficiency anemia in some patients[102]. Other authors, based on the co-localization of hepcidin with insulin-storing secretory granules, suggested that regulation of iron and glucose metabolism are distinctly coupled at the pancreatic level by the co-release of insulin and hepcidin[108].

CONCLUSIONS

Despite these recent important advances, much work still needs to be done. For example, the presence in blood of pro-hepcidin at concentrations greater than the iron active peptide remains puzzling. Huang *et al*[115] recently suggested that pro-hepcidin conversion into hepcidin may occur in the circulation as part of an iron-dependent regulation at the post-translational level, but this needs to be confirmed in larger studies. Other issues to be clarified are fluctuations of hepcidin related to circadian rhythm and/or meals[72,76], and if urinary hepcidin quantification can give information that might be complementary to single point serum measurement, similar to many other hormones. Since hepcidin is directly implicated in the regulation of iron homeostasis, its measurement might turn out to be a useful tool in the differential diagnosis of iron overload disorders and iron deficiency. The definition of the biological value of the hepcidin/ferritin ratio will be important to understand the appropriateness of hepcidin production in the clinical setting. Similarly the response of hepcidin to oral iron might prove to be a useful test to evaluate iron absorption in iron deficiency and the iron-sensing pathway in iron overload and in targeting phlebotomy treatment in patients with hemochromatosis[20]. Finally, of special importance are collaborative studies among various laboratories aimed at comparing the analytical performance of different methods, as well as for promoting standardization of hepcidin assay. In this regard, the results of the ongoing first international round robin for quantification of urinary and plasma hepcidin (Kroot *et al*, *Blood Suppl.* in press) are eagerly awaited.

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ORIGINAL ARTICLES

Hepatic non-parenchymal cells and extracellular matrix participate in oval cell-mediated liver regeneration

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Abstract

AIM: To elucidate the interaction between non-parenchymal cells, extracellular matrix and oval cells during the restituting process of liver injury induced by partial hepatectomy (PH).

METHODS: We examined the localization of oval cells, non-parenchymal cells, and the extracellular matrix components using immunohistochemical and double immunofluorescent analysis during the proliferation and differentiation of oval cells in N-2-acetylaminofluorene (2-AAF)/PH rat model.

RESULTS: By day 2 after PH, small oval cells began to proliferate around the portal area. Most of stellate cells and laminin were present along the hepatic sinusoids in the periportal area. Kupffer cells and fibronectin markedly increased in the whole hepatic lobule. From day 4 to 9, oval cells spread further into hepatic parenchyma, closely associated with stellate cells, fibronectin and laminin. Kupffer cells admixed with oval cells by day 6 and then decreased in the periportal zone. From day 12 to 15, most of hepatic stellate cells (HSCs), laminin and fibronectin located around the small hepatocyte nodus, and minority of them appeared in the nodus. Kupffer cells were mainly limited in the pericentral sinusoids. After day 18, the normal liver lobule structures began to recover.

CONCLUSION: Local hepatic microenvironment may participate in the oval cell-mediated liver regeneration through the cell-cell and cell-matrix interactions.

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Key words: Oval cells; Liver regeneration; Extracellular matrix; Hepatic stellate cells; Kupffer cells

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INTRODUCTION

The capacity of hepatocytes and cholangiocytes to contribute to their own maintenance has long been recognized. They transiently enter the cell cycle and proliferate to restore the lost liver mass after parenchymal damage[1,2]. If the proliferation of hepatocytes is inhibited or blocked (such as by viral infection or chemicals), or if liver damage is extensive and chronic, putative liver progenitor cells are activated to proliferate into mature liver cells[3-7]. Liver progenitor cells are evident in both human pathologic ductular reactions and experimental animal models during hepatocarcinogenesis or as a part of the restituting response to liver injury[8-10]. After liver injury was induced by N-2-acetylaminofluorene (2-AAF)/partial hepatectomy (PH) in rodent models, the best candidate for liver progenitor cells would be the "oval cell" population, which is oval in shape, with a darkly staining nucleus and scant, basophilic cytoplasm. These cells radiate out from terminal biliary ductules, proliferate and migrate from portal regions into the parenchyma until liver regeneration is completed.

In recent years, attention has focused on the influence of the hepatic microenvironment on hepatic oval cell activation and proliferation[11-13]. The microenvironment comprises the extracellular matrix, epithelial and non-

epithelial resident liver cells, and recruited inflammatory cells as well as the variety of growth-modulating molecules. It is conceived as a restricted locale in an organ that regulates liver progenitor cell division through microenvironmental signaling, supporting their self-renewal, inhibiting or maintaining normative baseline differentiation in normal physiological states, and promoting proliferation and differentiation in response to injury[14-16]. The modulation factors for oval cell proliferation and differentiation are now being identified. Although great progress has been made in the research of multiple growth modulators (priming factors, growth factors, inflammatory cytokines, chemokines and growth inhibitory factors) involved in oval cell regulation[13], the role of individual non-parenchymal cells and hepatic extracellular matrix in oval cell-mediated liver regeneration remains unclear.

In the present work, we present a detailed immunohistochemical analysis of the involvement of non-parenchymal cells, hepatic extracellular matrix components and the interaction of these elements with oval cell and hepatocytes in oval cell-mediated liver regeneration induced by partial hepatectomy after 2-AAF treatment. To obtain detailed morphological assessment, the samples were analyzed under traditional light microscopy and confocal microscopy. The results indicated that different non-parenchymal cells and extracellular matrix component proliferation and migration were accompanied with the "ductular" periportal oval cell response. The local hepatic microenvironment may influence the oval cell response through the production of growth factors, expression of growth factor receptors and remodeling the hepatic extracellular matrix during the restitution process.

MATERIALS AND METHODS

Animal models

Male Sprague Dawley (SD) rats (8-9 wk of age; 120-150 g of body weight) were used. They were fed standard pelleted chow and had access to water *ad libitum*. Rats were maintained in a temperature-controlled room with a 12-h light/dark illumination cycle. To inhibit hepatocyte proliferation, all rats received daily oral gavage of 2-AAF (Sigma Chemical Co) at a dosage of 15 mg/kg body weight for 4 d before and up to 7 d after PH. 2-AAF was dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co). After the first four daily gavages, all rats were anesthetized, and two-thirds partial hepatectomy was performed by surgical removal of the left and median liver lobes; no dosing was performed on the day of surgery. Three rats were killed at 2, 4, 6, 9, 12, 15, 18 and 21 d after PH. Formalin-fixed and paraffin-embedded serial liver tissue sections (4 μm) were used for immunohistochemical and double immunofluorescent analysis. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

Immunohistochemistry

Paraffin sections of formalin-fixed liver tissues were stained with mouse monoclonal antibody to ov-6 (MAB2020, R&D Systems, Inc), a marker of hepatic oval cells in ductular reaction; mouse monoclonal antibody to desmin (DE-R-11, DAKO Denmak), a marker of stellate (Ito) cells, smooth muscle cells, periportal fibroblasts; mouse monoclonal antibody to ED1 (MCA341GA, Serotec Ltd) recognizing the cytoplasmic antigen in circulating monocytes and small Kupffer cells in the liver; and polyclonal rabbit anti-laminin (Z0097, DAKO, Denmark) or polyclonal rabbit anti-fibronectin (A0245, DAKO Denmark). Tissue sections were rehydrated at descending concentrations of ethanol and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Tissues used for ov-6 and desmin immunohistochemistry were microwaved to boiling for 15 min in 10 mmol/L Tris buffer, 1 mmol/L ethylenediamine tetra-acetic acid (EDTA), pH 9.0, for antigen retrieval. The sections labeled ED1, laminin or fibronectin were digested with Proteinase (10 μg/L, 5 min, 37℃). After antigen retrieval, the tissue sections were blocked with 10% normal serum from the donor species of the secondary antibodies for 15 min at room temperature, followed by incubation with primary antibodies overnight at 4℃. Primary antibody dilutions were as follows: anti-ov-6, 1:10; anti-desmin, 1:50; anti-ED1, 1:50; anti-laminin, 1:100 and anti-fibronectin, 1:50. After rinsing with phosphate buffered solution (PBS), primary antibodies were detected by incubation for 30 min with biotinylated rabbit anti-mouse or goat anti-rabbit immunoglobulins. After further rinsing with PBS, sections were incubated with horseradish peroxidase conjugated streptavidin/biotin complex (85-9843, Histostain Plus Kits, Zymed Laboratories Inc.). Peroxidase activity was developed with 0.05% diaminobenzidine and 0.03% H_2O_2. Finally, sections were counterstained for 5 min in hematoxylin, dehydrated through graded alcohols, and mounted under glass coverslips.

Double immunofluorescent analysis

After deparaffinization and hydration, liver sections were microwaved for 15 min in 10 mmol/L Tris buffer, 1 mmol/L EDTA, pH 9.0, for antigen retrieval. Proteinase digestion was added if the second primary antibodies were laminin, ED1 or fibronectin. Five pairs of double labeling (ov-6/desmin, ov-6/laminin, ov-6/ED1, ov-6/fibronectin, desmin/laminin) were performed. We used a vector Mouse-On-Mouse (MOM) immunodetection kit (FMK2201, Vector Laboratories Inc.) when both primary antibodies came from mice. After rinsing with PBS, tissue sections were incubated for 5 min in working solution of MOM™ diluent, followed by incubation with primary antibodies overnight at 4℃. Working solution of MOM biotinylated anti-mouse IgG was used as secondary antibodies. Then, sections were incubated with fluorescein avidin DCS for 5 min. Before incubating

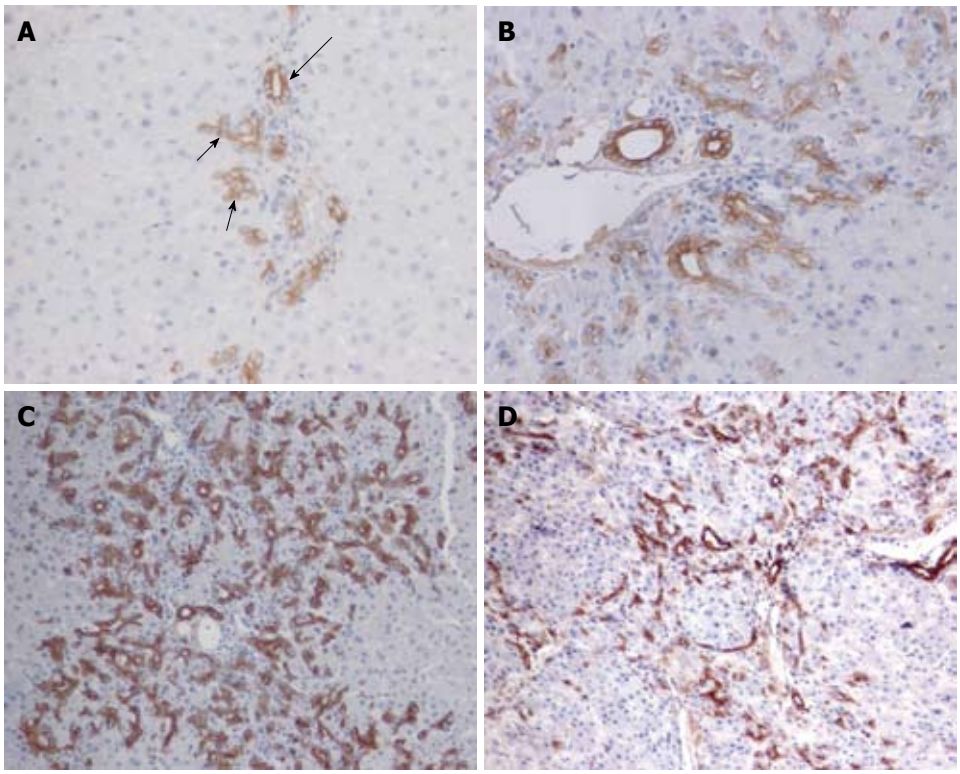

Figure 1 Ov-6 immunoreactivity of ductular cell reaction following PH of rats treated with AAF. A: Oval cells (short arrow) close to hepatocytes at limiting plates on day 2 after PH show much lighter labeling than pre-existing bile duct cells (long arrow) (× 200); B: On day 4 after PH, strongly stained cells are beginning to move into the parenchyma (× 200); C: On day 9 after PH, apparent long cords of ductular oval cells are fanning outward from each portal area (× 100); D: On day 15, the ov-6 (+) ductular structures are restricted to the periphery of small hepatocyte nodus (× 100).

the second primary antibodies, both the avidin/biotin blocking step and the mouse Ig blocking step must be done to prevent the interaction of the second set of labeling reagents with the first set of labeling reagents if the two mouse monoclonal primary antibodies were used. If two primary antibodies came from different species, only the avidin/biotin blocking step was necessary. After the protein blocking step, sections were incubated with the second primary antibodies for 1 h at 37℃, followed by incubation with working solution of MOM biotinylated anti-mouse or anti-rabbit IgG (BA-1000, Vector Laboratories Inc.) for 10 min, then Texas Red Avidin DCS (A-2016, Vector Laboratories Inc.) for 10 min. All samples were analyzed by confocal laser-scanning microscopy using the Nikon Digital ECLIPSE C1 system (Nikon Corporation, Japan).

RESULTS

Ductular oval cell response

In the normal liver, ov-6 strongly labeled pre-existing intraportal bile ducts, ductules and terminal duct cells. There was no change from normal in the distribution and the expression pattern of the ov-6 (+) cells on day 1 after PH. On day 2 after PH, small cells with high nuclear/cytoplasmic ratio (oval cells) were evident in and around the portal area. Oval cells were close to hepatocytes at limiting plates (Figure 1A). On day 4 after PH, ov-6 positives cells could be seen protruding into the parenchyma. Pre-existing ducts stained more strongly than new ducts (Figure 1B). By day 6, there is further extension of ductular structures across the hepatic lobule. On day 9 after PH, multiple strings of ductular cells spread further into the midzone (Figure 1C). By day 12 and beyond, the periportal areas were colonized by small hepatocyte nodus, though small strings of ductular cells could still be discerned at the periphery of hepatocytes. Many ductular profiles exhibited what looked like clear intestinal differentiation. On day 15 after PH, there was a significant decrease in the ductular structure and increase in the new small hepatocyte nodus (Figure 1D). From day 18 to 21 after PH, small ov-6 (+) cells further decreased or disappeared. The normal liver lobule structures recovered.

Non-parenchymal cell response and interaction with oval cells

Desmin stained smooth muscle cells of the blood vessels and dendritic cells around the bile ducts and vessels, but not sinusoidal cells as in normal rat liver. However, by day 2 after PH, hepatic stellate cells (HSCs) were activated and expressed desmin. At this moment, a few oval cells began to proliferate from the portal areas. Most desmin (+) cells were located in the portal areas and minority of the HSCs were small interlobular sinusoidal cells (Figure 2A). There were no desmin (+) cells in the central zone. From day 4 to 6 after PH, desmin (+) cells could be seen protruding into the parenchyma and formed the organized meshwork arrangement (Figure 2B). As time elapsed, HSCs spread further into the parenchyma and reached the mid zonal parenchyma. The number of desmin (+) periportal stellate cells peaked on day 9 and 12 after PH and decreased afterward. During the course of proliferation, HSCs appeared to be closely associated with the oval cells. They appeared to admix with oval cells in the periportal zone and tightly surrounded the long strings of ductular oval cells (Figure 2C). Along with the formation of the small

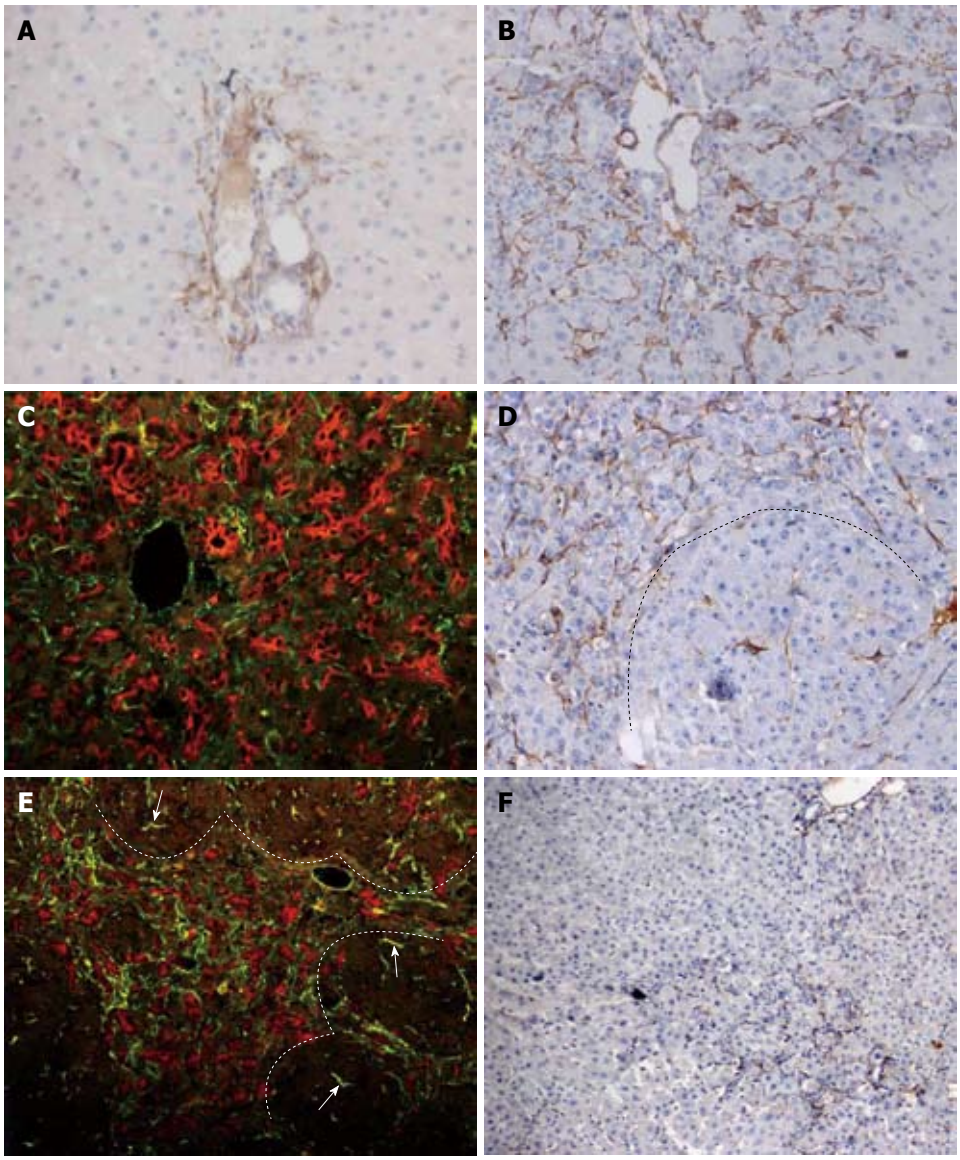

Figure 2 HSCs response and correlation with oval cell following PH of rats treated with AAF. A: Increase in number of desmin positive stellate cells in the portal areas. Very few of HSCs were small interlobular sinusoidal cells on day 2 after PH (× 200); B: Further increase in number of stellate cells at the periphery of the portal areas at day 6 after PH (× 200); C: Double immunofluorescent labelling for ov-6 (red) and desmin (green) on day 9 after PH. The strings of ductular oval cells are closely surrounded by the mesh-like desmin (+) stellate cells (× 400); D: On day 12 after PH, dash line marks the edge of a regenerative small hepatocyte focus. Desmin (+) cells are present around the focus, and occasionally in the focus (× 200); E: Double immunofluorescent labelling for ov-6 (red) and desmin (green) on day 12 after PH. Desmin (+) cells accompany the ductular structure around small hepatocytes focus. There are few HSCs in the focus negative for ov-6 (× 200); F: By day 18 after PH, the number of desmin (+) portal stellate cells is further decreased (×100).

hepatocyte nodus, most of the desmin (+) cells were located around the nodus. There were few activated HSCs in the nodus (Figure 2D). In addition, increasing numbers of desmin (+) cells were seen in the interlobular sinusoidal zone. On day 15 after PH desmin (+) cells became far less prominent and accompanied with the ductular structure around small hepatocyte nodus (Figure 2E). The number of stellate cells decreased more significantly than the ductular oval cells. On day 18 after PH, the number of desmin (+) stellate cells further decreased or vanished completely (Figure 2F).

ED1 stained the small rounded or spindle-like macrophages in the sinusoids with a cytoplasmic staining pattern in normal liver (Figure 3A). The number of ED1 (+) cells sharply increased not only in the pericentral zone but also in the periportal zone by day 2 after PH. At this time, no obvious ductular structures spread into the parenchyma (Figure 3B). On day 4, ED1 (+) cells began to decrease in both portal and central zones with an increase of the ductular oval cells. Infiltration of ED1 labelled macrophages into the ductular structures was seen in the periportal areas. A few scattered ED1 (+) cells around the portal zone appeared to admix with oval cells (Figure 3C and D). From day 6 to 9, there appeared to be different fate for central zone and portal zone macrophages. The number of ED1 (+) cells in the periportal zone decreased more markedly than in the pericentral zone as the ductular oval cells spread further into the mid zone (Figure 3E). On day 12, ED1 staining was limited to a few pericentral sinusoidal macrophages. There were very few ED1 (+) cells in the expanding ductular cells (Figure 3F). After day 15, ED1 (+) cells were seen mainly located between the central veins and small hepatocyte nodus as oval cells differentiated into small hepatocyte-like cells.

Extracellular matrix changes and interaction with oval cells

Laminin normally stained around the bile ducts, blood vessels, and in the sinusoids. On day 2 after PH, laminin appeared along the hepatic sinusoids in the peirportal areas and in the cytoplasm of few nonparenchymal cells, as well (Figure 4A). During the course of oval cell proliferation, laminin-containing basement membrane

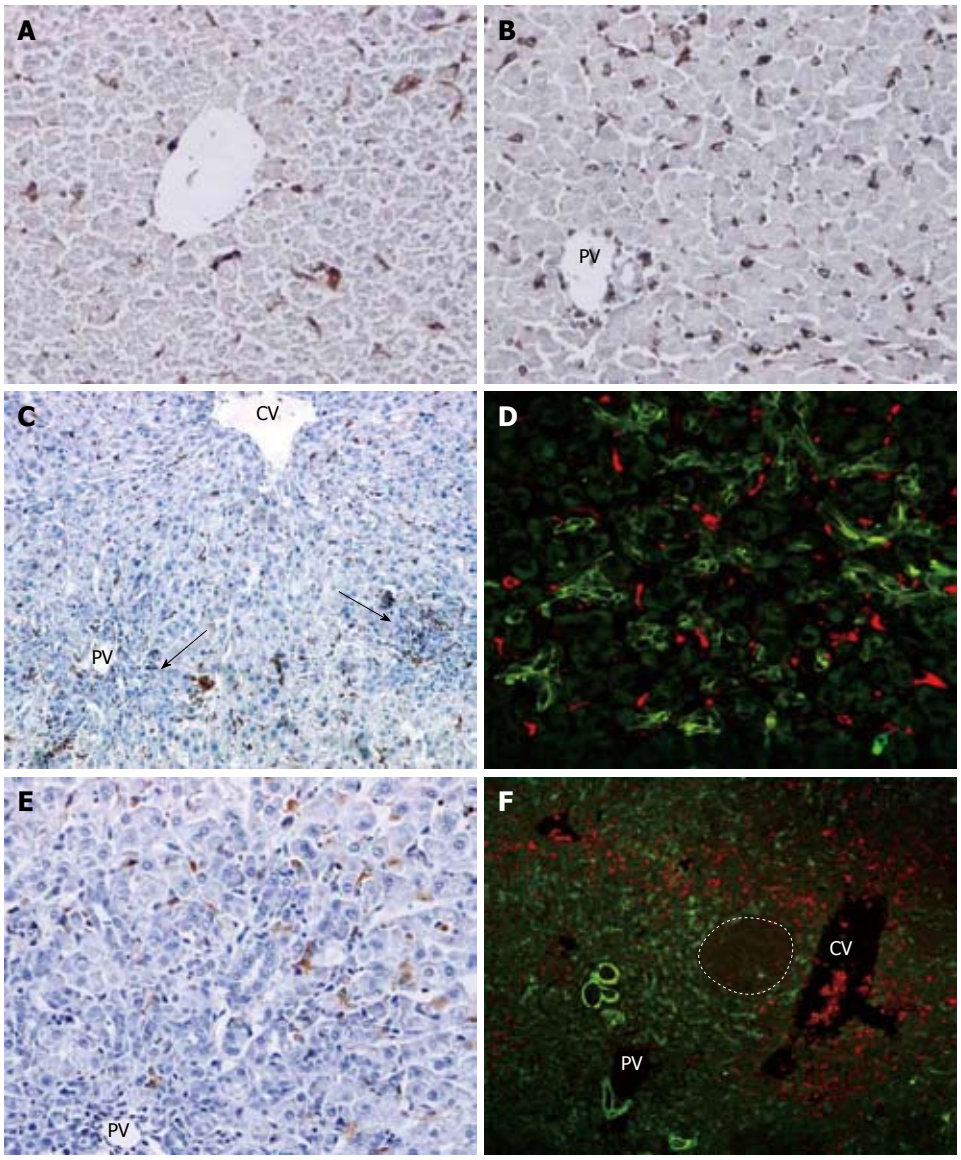

Figure 3 Kupffer cell response and correlation with oval cells following PH of rats treated with AAF. A: Normal rat liver. Small round or spindle-like cells in the sinusoids are labelled (× 200); B: On day 2 after PH, there is marked increase of ED1 (+) cells in periporal and interlobular sinusoids. No oval cells are seen in the poral areas (× 200); C: on day 4 after PH, number of pericentral and periportal ED1 (+) cells decreased along with the ductular oval cells(arrows) (× 100); D: double immunofluorescent labelling for ov-6 (green) and ED1 (red) at day 4 after PH. A few scattered ED1 (+) cells around the portal appeared to admix with ov-6 (+) oval cell (× 400); E: on day 9 after PH, very few ED1 (+) cells are visualized in the periportal ductular oval cells (× 200); F: double immunofluorescent labelling for ov-6 (green) and ED1 (red) on day 12 after PH. Dash line marks the edge of a regenerative small hepatocyte nodus. ED1 (+) cells are situated around the small hepatocte nodus and ductular ov-6 (+) cells (× 100).

surrounded the undifferentiated oval cells (Figure 4C). The cylinder formed by the basement membrane had an open end plugged by hepatocytes (Figure 4B), and desmin-positive stellate cells were accompanied with the ductular structures outside the basement membrane. In addition, laminin was found in the cytoplasm of many desmin (+) fusiform stellate cells (Figure 4D). No cells penetrating through the basement membrane could be observed. The maximum laminin staining was from day 9 to 12 after PH and decreased afterwards. The paucity or complete lack of laminin staining was characteristic of nodus as oval cells differentiated into small hepatocyte nodus on day 12 after PH. Numerous undifferentiated oval cells located outside nodus were still outlined brightly by laminin staining. After day 15, the ductular oval cells further decreased and the small hepatocyte-vascular relationship and hepatic architecture began to be restored. Straps of laminin were present in the periportal hepatocyte cluster in which there was no intervening sinusoids or extracellular matrix (ECM) in the early stage of regeneration (Figure 4E).

In normal liver, fibronectin is present in the perisinusoidal space and codistributed with collagens in the ECM of the subcapsular region, portal triad, and central vein regions. In general, the cellular staining for fibronectin in the normal liver is very similar to that of ED1. On day 2 after PH, fibronectin staining became more prominent in sinusoidal cells and in the periportal and pericentral regions (Figure 5A). By day 4, fibronectin (+) cells increased further in the periportal areas accompanying with ductular oval cells as oval cells began to spread into the parenchyma, and there was a marked decrease of fibronectin staining in the sinusoids of the mid zone and the pericentral areas (Figure 5B). From day 6 to 9, fibronectin admixed with the proliferating ductual oval cells expanded further into the pericentral areas. The clumps of oval cells were closed associated with desmin, and fibronectin as well (Figure 5C). On day 12, fibronectin (+) cells were seen around the small hepatocyte nodus in the periportal areas and very little fibronectin was detected within the hepatocyte clusters (Figure 5D). The number of fibronectin (+) cells among the expanding ductules decreased as oval

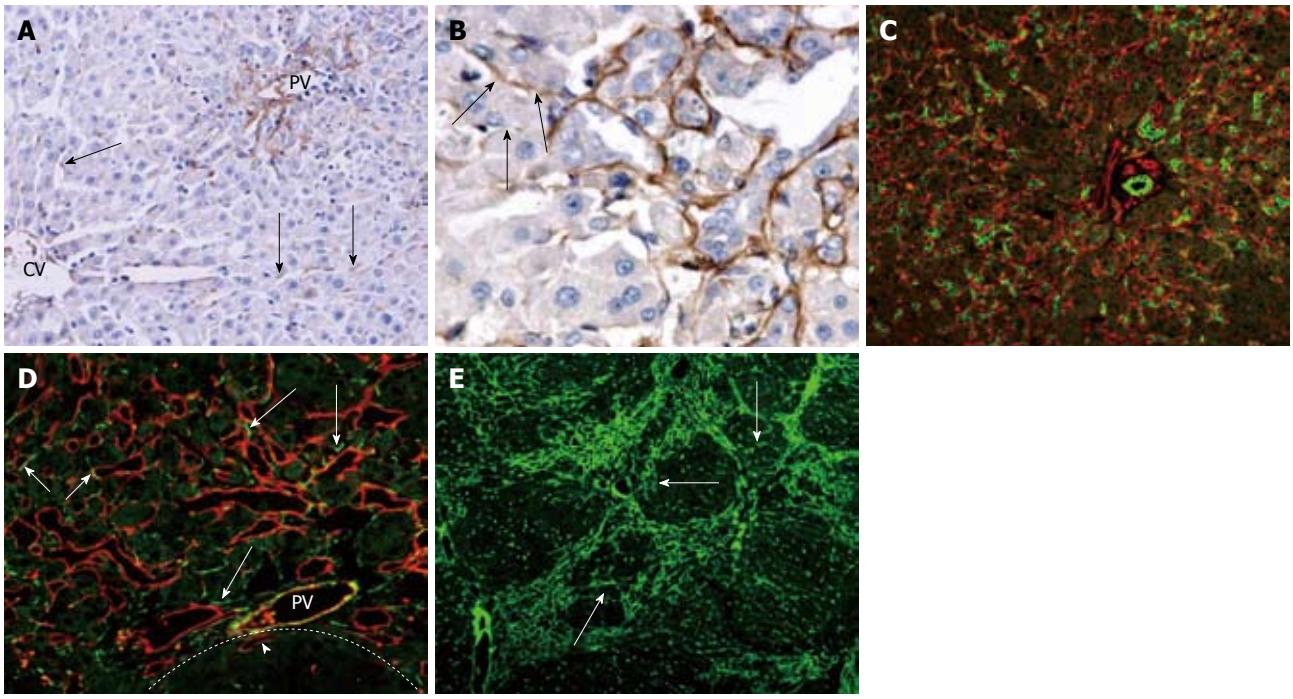

Figure 4 Laminin distribution and correlation with oval cell following PH of rats treated with AAF. A: On day 2 after PH, laminin is present along hepatic sinusoids in peirportal areas and in the cytoplasm of few nonparenchymal cells in the lobule (arrows) (× 200); B: On day 6 after PH, laminin positivity around the ductular oval cells. The proximal part of the ductule has continuous laminin staining, whereas distally the basement membrane is fragmented or absent (arrows) (× 400); C: Periportal area stained for OV-6 (green) and laminin (red) 12 d after PH. The bifurcating ductule strongly positive for OV-6, is surrounded by continuous basement membrane (× 200); D: Portal area 15 d after PH, stained for laminin (red) and desmin (green). Some of the desmin-positive cells are positioned closely to the laminin (+) basement membrane of ductules (long arrows). Some of the laminin-positive ductules also stained positively for desmin (short arrows). The focus (dash line marks the edge) is negative for laminin except some oval cell ductules enclosed in the focus (arrow head) (× 400); E: On day 15 after PH, most laminins are present around the focus, some laminins invading the hepatocyte clusters (arrows) (× 100).

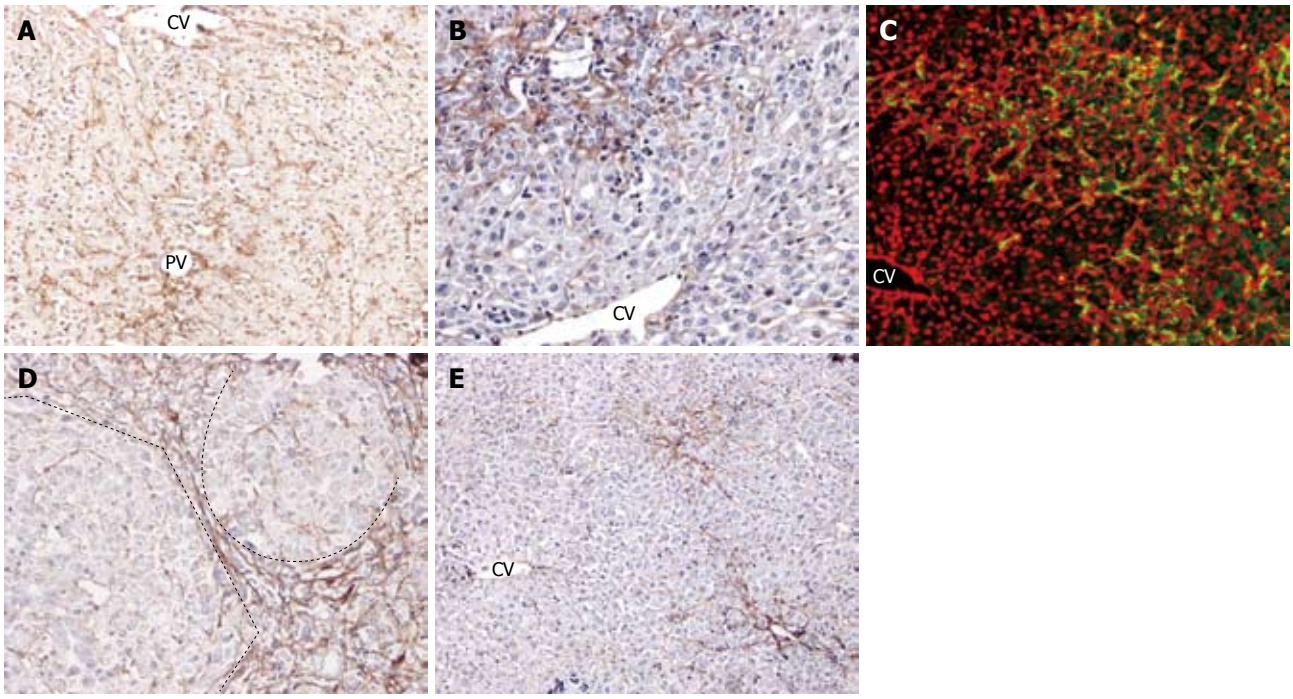

Figure 5 Fibronectin distribution and correlation with oval cell following PH of rats treated with AAF. A: On day 2 after PH, there is a marked increase of fibronectin in the periportal, pericentral areas and interlobular sinusoids (× 100); B: On day 4 after PH, there is a decrease in number of fibronectin in pericentral zone and increase of fibronectin in periportal areas (× 200); C: Double immunofluorescent labelling for ov-6 (green) and fibronectin (red) on day 9 after PH. The ductular oval cells fanning outward from portal area were closely surrounded by fibronectin (× 200); D: On day 12 after PH, dash line marks the edge of a regenerative small hepatocyte focus. Fibronectin was present around the focus, very few in the nodus (× 200); E: On day 15 after PH, there is notable decrease of fibronectin during recovery of normal hepatic architecture (× 100).

cells differentiated into the hepatocytes on day 15 (Figure 5E). Recovery of normal sinusoidal fibronectin expression occurred after day 18.

DISCUSSION

In this paper, we describe the proliferation of non-parenchymal cells and extracellular matrix components in the oval cell-mediated liver regeneration in a 2-AAF/PH rat model. In addition, the interaction of these elements with oval cell and hepatocytes was studied through confocal double immunofluorescent labeling. Our results show that the reconstitution of the liver parenchymal following 2-AAF/PH treatment involves not only the ductular oval cells but the Kupffer cells, hepatic stellate cells and ECM components (laminin and fibronectin). A close anatomical relationship among the hepatic oval cells, non-parenchymal cells and ECM was observed during the restituting process. These results suggest that ECM remodeling and production of growth factors and expression of growth factor receptors by non-parenchymal cells play an important role in the oval cell-mediated liver regeneration.

The effect of HSCs on oval cell proliferation and differentiation is complex, including elaboration and secretion of specific paracrine stimuli to proliferation in the early phase after partial hepatectomy, inhibition of proliferation in the later phase and matrix remodeling. During oval cell growth and ductule formation, HSCs are closely associated with oval cells, as suggested by Alison *et al*[17]. Both HSCs and oval cells produce and have receptors for a variety of overlapping growth factors[18]. Liver stem-like cells can differentiate into hepatocytes induced by coculture with hepatic stellate cells. These results suggest that, besides growth factors, cell-cell interaction through extracellular matrices produced by HSCs is also important for the induction of hepatocytic differentiation[19]. Another important role of HSCs is matrix remodeling by combination of proteolytic degradation of ECM and extracellular matrix synthesis. HSCs exhibit collagenase, stromelysins, metalloelastase and gelatinase A activity. Tissue inhibitors of metalloproteinases, also HSC derived, balance these activities. Matrix components, including laminins, fibronectins, collagens and proteoglycans, are largely but not exclusively HSC derived[20]. Our results show that after ductular oval cells differentiate into small hepatocyte nodus, the HSCs extend into the hepatocyte clusters without intervening sinusoids or ECM. HSCs gradually disappear as the normal liver lobule structures are restored. These results indicate that HSCs may play an important role in the restoration of normal hepatocyte-vascular relationships and overall hepatic architecture in the later stages of regeneration.

Concomitant with periportal oval cell proliferation, there is an increase in ED1 (+) Kupffer cells. Kupffer cells are mainly phagocytic cells also synthesize and secrete a number of cytokines, including tumor necrosis factor-α (TNF-α), IL-1 and IL-6[20-22]. Inhibition of TNF signaling results in a reduced progenitor cell response and impaired liver regeneration[23]. In our study, there was a quick increase in ED1 (+) Kupffer cells in the whole hepatic lobule by day 2 after PH, before obvious ductular structures were observed. From these results, we can speculate that Kupffer cells may play a crucial role in priming the oval cells and inducing DNA synthesis by secreting the priming factors (TNF-α and IL-6) in the early phase of oval cell-mediated liver regeneration. After 4 d, as the oval cells spread further into the liver parenchyma, Kupffer cells appeared to admix with the expanding oval cell population. The structural relationship of the interaction of the responding cells implies that there may also be a functional interaction. Takeishi *et al*[24] demonstrated that Kupffer cells play a stimulatory role in liver regeneration by enhancing hepatocyte growth factor (HGF) expression. In addition, studies indicate that HGF accelerates the proliferation of hepatic oval cells and promotes the differentiation to hepatocytes[25,26]. Apart from secreting growth factors, Kupffer cells may also produce metalloproteases, elastase, collagenase and fibronectin[27]. Thus, Kupffer cells and HSCs may act together to contribute to the dissolution of basement membrane around small ductules, perhaps allowing ductular oval cells to move into the adjacent hepatic lobe, providing mitogenic growth factor and remodeling ECM during the proliferation and differentiation of oval cells. Kupffer cells also play a role in terminating the surge of replication after partial hepatectomy[20]. One of powerful inhibitors of hepatocyte replication is TGF-β, produced most prominently by hepatic stellate cells and Kupffer cells. TGF-β has been proposed to play similar modulatory roles in oval cell-mediated liver regeneration[28].

The extracellualr matrix is a dynamic complex of macromolecules and plays a role not only in structural support, but also in cell proliferation, migration, and differentiation[29]. The morphological observations described above suggest a link between stroma in the hepatic microenvironment and the oval cell response. We speculate that ECM (laminin and fibronectin) may affect the oval cell response by: (1) Providing the growth factors for oval cells in the early stage of regeneration. The upregulation of urokinase-type plasminogen activator (uPA) mRNA accompanying oval cell proliferation has been reported, and infusion of uPA enhanced the mitogenic response of cells located near bile ducts[30]. uPA has been shown to initiate the degradation of the ECM through activation of metalloproteinase and the degradation leads to the release of bound HGF with a subsequent increase in serum HGF concentration[31]. Several growth factors involved in oval cell regeneration may be also regulated by this system[18]. Together, these findings support the overall concept of ECM remodeling as an important step in the growth phase of oval cell-mediated liver regeneration. (2) Influencing the differentiation state of the oval cell. Our results show that there is a distinct

continuous laminin staining around the oval cells, and the disappearance of the basement membrane (BM) that surrounds the oval cell ductules is closely associated with initiation of the differentiation into hepatocytes. Yin *et al*[32] reported that isolated hepatic stem cells expressed biliary or hepatocytic phenotypes in culture, depending on the presence or absence of basement membrane matrix (Matrigel). Matrigel was also found to play an important role in maintaining the biliary phenotype in tissue culture in other experimental systems[33,34]. Recently Leite *et al*[35] demonstrate that fibronectin and laminin can induce expression of islet cell markers in hepatic oval cells in culture. (3) Affecting the migration, proliferation and attachment of oval cells. Sánchez *et al*[36] demonstrate that fibronectin might regulate morphology, cell organization and gene expression of rat fetal hepatocytes in primary culture. Other studies show that the apparent affinity of hepatocytes to laminin increases during the prereplicative phase of rat liver regeneration and laminin is one of the most effective substrates in supporting the responstiveness of hepatocytes to the growth stimulus[37,38]. (4) Playing a role in the development of the hepatic sinusoidal vasculature. We observed that both laminin and fibronectin are present in the periportal hepatocyte cluster and when the maturation of the sinusoidal vasculature is complete, the expression is repressed. A resynthesis of the previously degraded perisinusoidal ECM is required for full regeneration. Epithelial cells require contact with ECM to inhibit detachment-induced apoptosis, and the reconstitution of a perisinusoidal ECM therefore will stabilize the newly formed hepatocyte population[39].

In summary, our results indicate that there is a close relationship between the non-parenchymal cells (HSCs and Kupffer cells), ECM components (laminin and fibronectin) and oval cells during the restitutive repair in 2-AAF/PH model, providing evidence that the local hepatic microenvironment may participate in the oval cell-mediated liver regeneration through the cell-cell and cell-matrix interactions. Furthermore, our results also suggest that the production of growth factors and extracellular matrix remodeling may be required to regulate the migration, proliferation and differentiation of oval cells and the process of liver regeneration. Further studies should focus on providing the direct evidence of hepatic microenvironment in regulating to oval cells *via in vitro* experiments and elucidating the molecular mechanisms.

COMMENTS

Background

Oval cells are thought to be the progeny of stem cells in adult liver, which are able to differentiate bipotentially into mature hepatocytes and billary epithelial cells when the proliferation of mature hepatocytes is inhibited or blocked. In recent years, attention has focused on the influence of the hepatic microenvironment on hepatic oval cell activation and proliferation.

Research frontiers

Although great progress has been made in the research of multiple growth modulators involved in oval cell regulation, the role of individual non-parenchymal cells and hepatic extracellular matrix in oval cell-mediated liver regeneration remains unclear.

Innovations and breakthroughs

The current study demonstrated that the local hepatic microenvironment may influence the oval cell response through the production of growth factors, expression of growth factor receptors and remodelling the hepatic extracellular matrix during the restitution process.

Applications

By observing the interaction of hepatic microenvironment with oval cells in oval cell-mediated liver regeneration, the existence and importance of liver stem cell niche have been further confirmed. This study has laid a foundation for researchers to elucidate the molecular mechanisms of hepatic microenvironment in regulating oval cells.

Terminology

Stem cell niche is conceived as a restricted locale in an organ that regulates stem cell division through microenvironmental signaling, supporting their self-renewal, inhibiting or maintaining normative baseline differentiation in normal physiological states, and promoting proliferation and differentiation in response to injury.

Peer review

The authors reported a close anatomical relationship between the hepatic oval cells, non-parenchymal cells and extracellular matrix (ECM), and they suggested that ECM remodeling and production of growth factors and expression of growth factor receptors by non-parenchymal cells may play an important role in the oval cell-mediated liver regeneration. Overall, the data are well presented, although some conclusions could not be supported as this is only a immunohistochemical and a double immunofluorescent analysis.

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ORIGINAL ARTICLES

Cell-permeable Tat-NBD peptide attenuates rat pancreatitis and acinus cell inflammation response

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Abstract

AIM: To investigate the effects of Tat-NEMO-binding domain (NBD) peptide on taurocholate-induced pancreatitis and lipopolysaccharide (LPS)-stimulated AR42J acinus cells inflammatory response in rats.

METHODS: Sodium taurocholate (5%) was used to induce the pancreatitis model. Forty-eight rats from the taurocholate group received an intravenous bolus of 13 mg/kg Tat-NBD (wild-type, WT) peptide, Tat-NBD (mutant-type, MT) peptide, NBD peptide or Tat peptide. The pancreatic histopathology was analyzed by hematoxylin staining. LPS was added to the culture medium to stimulate the AR42J cells. For pretreatment, cells were incubated with different peptides for 2 h before LPS stimulation. Expression of *IL-1β* and *TNF-α* mRNA was analyzed using a semi-quantitative reverse-transcript polymerase chain reaction (RT-PCR) method. IL-1β and TNF-α protein in culture medium were detected by enzyme linked immunosorbent assay (ELISA). NF-κB DNA-binding in pancreas was examined by electrophoretic mobility shift assays. P65 expression of AR42J was determined by Strept Actividin-Biotin Complex (SABC) method.

RESULTS: Pretreatment with Tat-NBD (WT) peptide at a concentration of 13 mg/kg body wt showed beneficial effect in pancreaitis model. LPS (10 mg/L) resulted in an increase of *IL-1β* mRNA, IL-1β protein, *TNF-α* mRNA and TNF-α protein, whereas significantly inhibitory effects were observed when cells were incubated with Tat-NBD (WT). Consisting with p65 expression decrease analyzed by SABC method, NF-κB DNA-binding activity significantly decreased in Tat-NBD (WT) pretreatment group, especially at the largest dose. No significant changes were found in the control peptide group.

CONCLUSION: Our result supports that active NF-κB participates in the pathogenesis of STC-induced acute pancreatitis in rats. Tat-NBD (WT) peptide has anti-inflammatory effects in this model and inhibits the inflammation of acinus simulated by LPS.

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Key words: Pancreatitis; Nuclear factor kappa B; Cytokine; Peptide; Pretreatment

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INTRODUCTION

Because of the systematic inflammatory response syndrome (SIRS) in severe acute pancreatitis (SAP), the mortality rate of SAP is very high, reported to reach about 30%[1,2]. Though it is known that several kinds of proinflammatory mediators, such as lipopolysaccharide (LPS), cytokine, *etc.*, are closely related to the

development of systematic complications, the exact mechanism of acute necrotic pancreatitis is still unclear. Therefore, it is very important to develop an effective therapy. Nuclear factor kappa B (NF-κB) is a member of the Rel family of transcriptional regulatory proteins including p50, p52, p65, c-Rel and Rel B[3]. It has been proved that in many inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease and SIRS, NF-κB is highly activated while the inflammatory mediators, such as LPS, can intermediate the activation of NF-κB[4]. Once activated, NF-κB is translocated into the nucleus from the cytosol. It binds the consensus sequence to promoter or enhancer region of related genes and regulates gene transcription.

Activation of NF-κB dimers is regulated by the inhibitory proteins, IκBα, IκBβ, and IκBε. The phosphorylation sites of IκBα have been identified, and the kinases have been found involved in the process, namely the IκB kinase (IKK)[5,6]. The IKK complex comprised primarily three IKKs, α, β, and γ. IKKγ, the third protein within the IKK complex, which is also named NEMO (NF-B essential modulator), was deemed regulatory of the other IKKs. NEMO is critical for pro-inflammatory activation of the IKK complex[7,8]. Although the precise mechanism of NEMO action is poorly understood, it was speculated that it recruits the IKK complex to ligate cytokine receptors and facilitate transphosphorylation events. More intriguingly, NEMO may facilitate the recruitment of upstream IKK activators such as kinases that specifically target the activation loops within the catalytic domains of the IKK subunits. NEMO interacts with a COOH-terminal sequence within the both IKKs termed the NEMO-binding domain (NBD)[9]. Importantly, short cell-permeable peptide spanning the IKKβ NBD disrupted the association of NEMO with IKKβ, blocked NF-κB activation, and ameliorated responses in animal models of inflammation. These observations defined the IKK NBD as a viable target for the development of anti-inflammatory drugs[7,10,11]. Such peptide interacting the NBD had been confirmed to attenuate the severity of cerulein-induced mouse pancreatitis which ameliorated inflammation in the pancreas, reduced hemorrhage in the lungs, and lowered the myeloperoxidase activity in both pancreas and lung. It indicates that this novel compounds that selectively target NF-κB may be useful in the treatment of AP and AP-associated lung injury[12]. In rat pancreatitis induced by taurocholate, activation of NF-κB in pancreas has been reported[13]. It was not clear whether or not the peptide could have anti-inflammatory effects in taurocholate-induced SAP model and inhibited the inflammation of acinus stimulated by LPS directly. In this paper, we report the effects of Tat-NBD peptide on STC-induced pancreatitis and LPS-stimulated AR42J acinus cell inflammatory response in rats.

MATERIALS AND METHODS

Materials and animals

Sodium taurocholate and LPS were purchased from Sigma Company (America); AR42J cells, from American Type Culture Collection (ATCC N.O: CRL-1492); agarose, inhibitor of RNase and M-MLV retroviridase, from Promega Company (America); F12 culture medium, from Gibco Company (America); DNA marker, from Beijing Huamei Biological Company (China); primers for reverse transcription-polymerase chain reaction (RT-PCR) from Shanghai Boya Biological Company (China); TRIzolTM Reagent from Invitrogen Company (America); and EX Taq enzyme from TaKaRa Company (Japan). Peptide was synthesized by Xian Meilian Biological Company (China). TNF-α kit for enzyme-linked immunosorbent assay (ELISA) is a product of Guangzhou Jingmei Biological Company (China). Immunohistochemistry (IH) kit of steptavidin-biotin complex method (SABC) was obtained from Wuhai Boshide Company (China). Anti-rat monoclonal antibody of p65 is from Santa Crutz Company (America). All other chemicals were obtained from either Sigma or Beijing Dingguo Company. Female and male Sprague-Dawley (S-D) rats (200 ± 10 g) were from specific pathogeny free unit in the Animal Center of Guangdong Medical College.

Peptide synthesis

According to Dai *et al*[7], the wild Tat-NBD peptide was synthesized with a 23-amino acid sequence (YGRKKRRQRRR-G-TTLDWSWLQME). A mutant peptide was called Tat-NBD (MT) with TrpAla mutations (YGRKKRRQRRR-G-TTLDASALQME). NBD sequence (TTLDWSWLQME) and Tat sequence (YGRKKRRQRRR) were synthesized respectively as controls. The peptide concentrations of 0.1, 1, 10 and 100 mg/L were prepared with F12 culture medium (no serum and antibody).

Induced rat pancreatitis model

Seventy-two Sprague-Dawley rats were randomized into different groups. All rats were maintained at 23℃ on a 12 h light/dark cycle and allowed free access to water and standard laboratory chow. From 12 h before start of the experiments, the animals were deprived of food, except access to water. Acute taurocholate pancreatitis was induced according to Aho *et al*[14]. Under anesthesia with a combination of xylazine (10 mg/kg) and ketamine (100 mg/kg), a midline laparotomy was performed and a PE-50 catheter (inside diameter 0.58 mm, outside diameter 0.96 mm) was inserted in the pancreatic duct through a puncture of the duodenum. The biliopancreatic duct was occluded transiently by a silk ligature at the liver hilus to prevent regurgitation into the liver. Sodium taurocholate solution (1 mL/kg of 5%; STC group, n = 60) or the same volume of saline (sham-operated group, n = 12) was retrogradely infused in the pancreatic duct at a flow rate of 0.07 mL/min by a microinfusion pump. Upon the completion of the infusion, the catheter and hilar ligature were removed and the abdomen was closed with suture. For pretreatment with peptide, 48 rats from the TC group

received an intravenous bolus of 13 mg/kg Tat-NBD (WT) peptide [Tat-NBD (WT) group, n = 12], Tat-NBD (MT) peptide [Tat-NBD (MT) group, n = 12], NBD peptide (NBD group, n = 12) or Tat peptide (Tat group, n = 12). Peptide in F12 culture medium was incubated 2 h before intraductal infusion of taurocholate, followed by a continuous intravenous infusion. Controls received an intravenous saline infusion. These groups of rats were killed 6 and 12 h after taurocholate administration.

At 6 or 12 h after taurocholate infusion, animals were killed and blood was obtained with heparinized tubes for amylase and TNF-α determinations. Portions of the gland were saved for morphological examination, which were fixed in 10% zinc-buffered formalin, embedded in paraffin, and cut into 3 to 5 μm thick sections. The microscopic damage score was calculated according to Spormann *et al*[15].

Cell culture

The AR42J cell line was obtained from American Type Culture Collection. Cells were grown in 75-cm^2 flasks containing 12 mL medium, consisting of Ham's F-12 nutrient medium (F12K) with 2 mmol/L *L*-glutamine, 1% antibiotic, 1.5% sodium bicarbonate, and 10% FBS. Flasks were placed into an incubator maintained at 37℃ and a 5% CO_2-95% air atmosphere. Cells were plated at a density of 10^5 cells/mL in 12-well plates. LPS was added to culture media at a dose of 10 mg/kg for 2 h to stimulate the cells. For pretreatment, cells were incubated with different peptides [Tat-NBD (WT), n = 3; Tat-NBD (MT), n = 3; NBD, n = 3; Tat, n = 3] for 2 h before LPS stimulation.

AR42J, IL-1β and TNF-α mRNA detection by RT-PCR

Analysis of *TNF-α* mRNA expression was conducted by a semiquantitative RT-PCR method. Total RNA from cells was extracted using the TRIzol reagent (Invitrogen Life Technologies). One microgram of total RNA was used for amplification using the Invitrogen One Step RT-PCR System according to the manufacturer's instructions. The following primers were used for: *IL-1β* (307 bp) forward: 5'-GGATGATGACGACCTGCT AGTGT-3', reverse: 5'-CTTCTTTGGGTGTTTG GGA-3'; *TNF-α* (231 bp) forward: 5'-GAACTCCAG GCGGTGTCT-3', reverse: 5'-TCTGCTTGGTGGTTT GC-3'. Fragments were amplified using 25-30 cycles of PCR; each cycle consisted of 15 s at 94℃, 30 s at 55℃, and 1 min at 72℃. The resulting RT-PCR products were electrophoresed on 2% agarose gels with DNA markers, stained with ethidium bromide, and visualized under UV light. *Beta-Actin* (701 bp) was used as an internal control for stable expression (housekeeping gene) in all experiments. The forward primer was 5'-GCCAACCGT GAAAAGATGA-3', and the reverse primer was 5'-GCCAGGATAGAGCCACCAAT-3.

Electrophoretic mobility shift assays (EMSAs) of NF-κB

The activity of NF-κB DNA-binding was detected by EMSA as descried by Molloy[16] with the following modifications. The double-stranded oligonucleotide sequence of 5'-AGTTGAGGGGACTTTCCCAGGC-3, 3'-TCAACTCCCCTGAAAGGGTCCG-5', which corresponds to the κB binding site, was end-labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase (Promega). Labeled probes were purified and CPM detected with Whatman DE81 (> 5000 cpm/min). The nuclear extract equivalent to 15 μg was mixed with 5 × binding buffer 2 μL (room temperature, 10 min), then labeled probe (0.0175 pmoL) added for incubation (room temperature, 20 min). The mixture was subjected to electrophoresis on 4% polyacrylamide gel at 150 V in 0.5 × TBE buffer for 40 min at 4℃. After being dried, the gel was exposed to imaging system of FX P screen (Bio-Rad, America) for 24 h.

P65 expression of AR42J by SABC method

In immunohistochemical monitoring of NF-κB-p65 subunit to determine p65 expression, AR42J cells were incubated in coverslips overnight at 37℃ under 5% CO_2 in air. Cells were treated first with peptides or saline for 2 h, then LPS was added to the culture medium for 2 h. Cells were fixed with 4% formaldehyde in phosphate-buffered saline for 20 min at 4℃ and permeabilized with 0.1% Triton in phosphate-buffered saline for 5 min at room temperature. Saturation was obtained by incubating cells with 5% bovine serum albumin in phosphate-buffered saline for 30 min at room temperature. Cells were stained with anti-p65 NF-κB antibody (Santa Cruz). Staining step was referred to the SABC kit description. Slides' staining degree was scanned by imaging system (BIO-RAD) and semi-quantitated by software package of quantity.

IL-1β and TNF-α protein detection by ELISA

The detection was performed according to the manufacturer's instructions.

Statistical analysis

Parametric data were presented and statistical analyses were performed using two-way ANOVA with SPSS8.0. Results were considered significant when $P < 0.05$.

RESULTS

Pancreas histology

Histomorphology after sodium taurocholate-induced acute pancreatitis was analyzed semiquantatively using the Spormann score. Pancreatitis without treatment was characterized by severe interstitial edema formation, considerable necrosis of fatty tissue, distinct sublobular and lobular parenchymal necrosis, as well as hemorrhage, resulting in a Spormann score of 8.71 ± 0.45 at 6 h and 10.31 ± 1.23 at 12 h, which reflects severe, acute, necrotizing pancreatitis (Figure 1A and C). Pretreatment with Tat or Tat-NBD (MT) peptide at a concentration of 13 mg/kg body weight had no beneficial effect in the pancreatitis-associated tissue injury, whereas the same

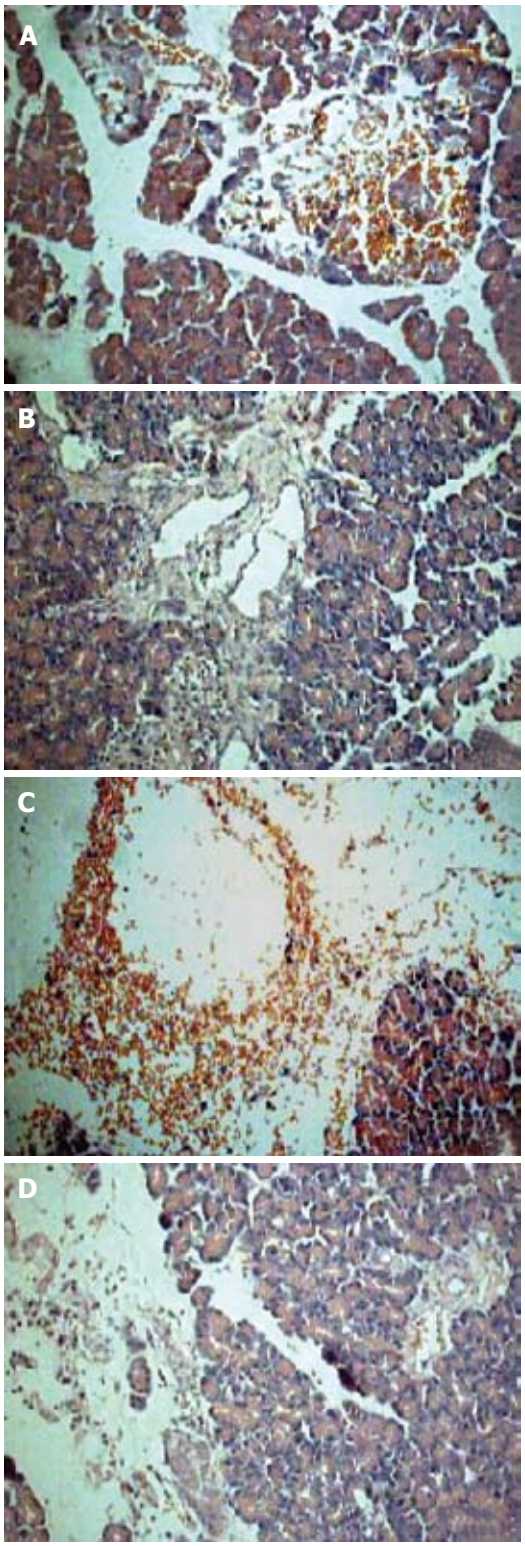

Figure 1 Tat-NBD attenuates STC-induced pancreatitis (HE, × 200). A: STC group at 6 h; B: Tat-NBD (WT) group at 6 h; C: STC group at 12 h; D: Tat-NBD (WT) group at 12 h.

concentration of NBD peptide caused a significant reduction of the tissue damage (Table 1). More beneficial effect can be seen in Tat-NBD (WT) group. The Spormann score was lowered to 5.04 ± 0.41 at 6 h and 5.45 ± 0.34 at 12 h, which was mainly due to a decrease in degree and amount of fatty tissue and parenchymal

Table 1 Pancreas histopathologic scores (mean ± SD)

Group	Histopathologic scores	
	6 h	12 h
Sham-operated	0	0
STC	8.71 ± 0.45	10.31 ± 1.23
Tat	9.84 ± 0.73	10.61 ± 1.62
NBD	6.76 ± 0.26[a]	8.31 ± 0.53[e]
Tat-NBD (WT)	5.04 ± 0.41[a]	5.45 ± 0.34[c]
Tat-NBD (MT)	8.85 ± 0.59[e]	10.13 ± 0.36[g]

[a]$P < 0.05$ *vs* STC group at 6 h; [c]$P < 0.05$ *vs* STC group at 12 h; [e]$P < 0.05$ *vs* Tat-NBD (WT) group at 6 h; [g]$P < 0.05$ *vs* Tat-NBD (WT) group at 12 h.

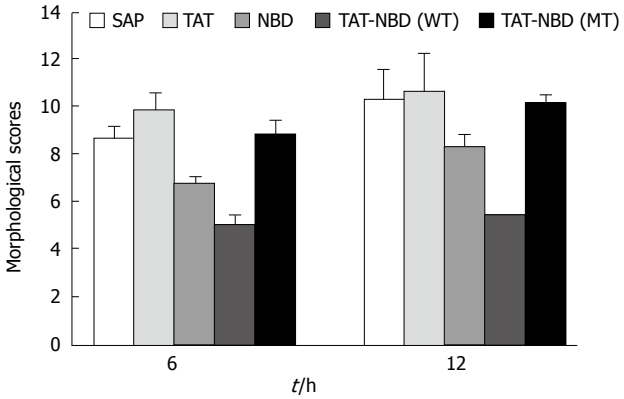

Figure 2 Pancreas histopathologic scores. The pretreatment of NBD peptide and Tat-NBD (WT) resulted in a significant reduction of the tissue damage score and more beneficial effect can be seen in the latter group at the two time points. Pretreatment with Tat or Tat-NBD (MT) peptide showed no therapeutic effect.

cell necrosis (Figure 1B and D). The histological scores in different groups are shown in Table 1 and Figure 2.

Cytokine change of AR42J

We used LPS (10 mg/L) to stimulate AR42J and explore whether the peptide could play a direct role in pancreatic acinus cells. Cells were cultured as previously stated.

IL-1β: LPS (10 mg/L) resulted in an approximately 10-fold increase of *IL-1β* mRNA, whereas significant inhibitory effects were observed when cells were incubated with Tat-NBD (WT) at a dose of 0.1 mg/L (Figure 3). The Tat-NBD (WT) peptide decreased IL-1β mRNA expression in a dose-dependent manner and its peak role appeared at a dose of 100 mg/L (Figure 4). We next determined whether Tat-NBD (WT) peptide attenuated IL-1β protein, which was activated by LPS. Culture medium was analyzed by ELISA. Results confirmed that IL-1β protein was inhibited by Tat-NBD (WT) at different doses, which also presented in a dose-dependent manner. Finally, we investigated whether the control peptide of NBD, Tat or Tat-NBD (MT) could block LPS-induced *IL-1β* mRNA or protein increasing in AR42J cells. Cells were preincubated for 2 h at a concentration of 10 mg/L of Tat-NBD (WT) or control prior to stimulation with LPS. Cells were harvested 120

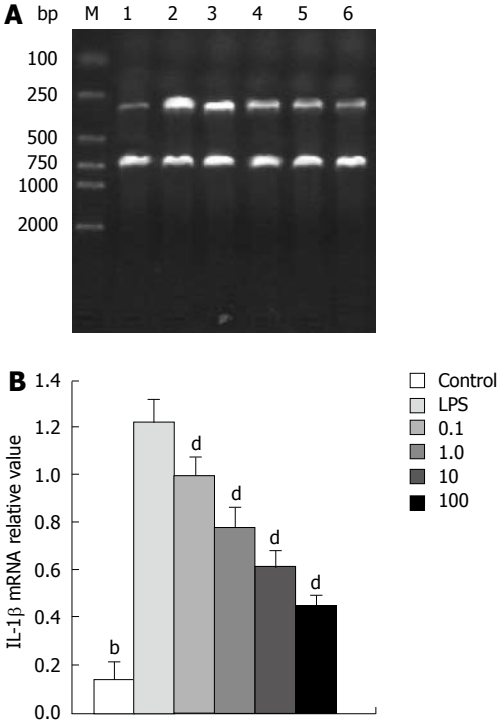

Figure 3 Effects different doses of Tat-NBD (WT) in *IL-1β* mRNA expression of AR42J by RT-PCR. A: Images of agarose gel electrophoresis; B: Tat-NBD (WT) peptide decreased *IL-1β* mRNA expression in a dose-dependent manner and its peak role appeared at a dose of 100 mg/L. Control: Cells were incubated with buffer control (n = 3); 1: Control; 2: LPS; 3: 0.1 mg/L Tat-NBD (WT); 4: 1 mg/L Tat-NBD (WT); 5: 10 mg/L Tat-NBD (WT); 6: 100 mg/L Tat-NBD (WT); M: Marker. LPS: Cells stimulated by LPS for 2 h (n = 3). 0.1: Pretreatment with 0.1 mg/L of Tat-NBD (WT) (n = 3); 1: Pretreatment with 1 mg/L of Tat-NBD (WT) (n = 3); 10: Pretreatment with 10 mg/L of Tat-NBD (WT) (n = 3); 100: Pretreatment with 100 mg/L of Tat-NBD (WT) (n = 3). $^{b}P < 0.01$ *vs* LPS group. $^{d}P < 0.01$ *vs* LPS group.

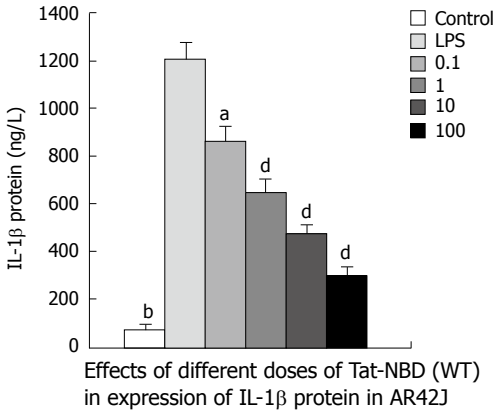

Figure 4 Effects of different doses of Tat-NBD (WT) in IL-1β protein expression of AR42J by ELISA. TAT-NBD (WT) decreased IL-1β protein in a dose-dependent fashion over a range of 0.1 to 100 mg/L. Control: Cells were incubated with buffer control (n = 3); LPS: Cells were stimulated by LPS for 2 h (n = 3). 0.1: Pretreatment with 0.1 mg/L of Tat-NBD (WT) (n = 3); 1: Pretreatment with 1 mg/L of Tat-NBD (WT) (n = 3); 10: Pretreatment with 10 mg/L of Tat-NBD (WT) (n = 3); 100: Pretreatment with 100 mg/L of Tat-NBD (WT) (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$, *vs* LPS group. $^{d}P < 0.01$ *vs* LPS group.

min after addition of LPS. RT-PCR and ELISA were performed as stated. Only Tat-NBD (WT), not the control peptides, inhibited IL-1β expression (Figures 5 and 6).

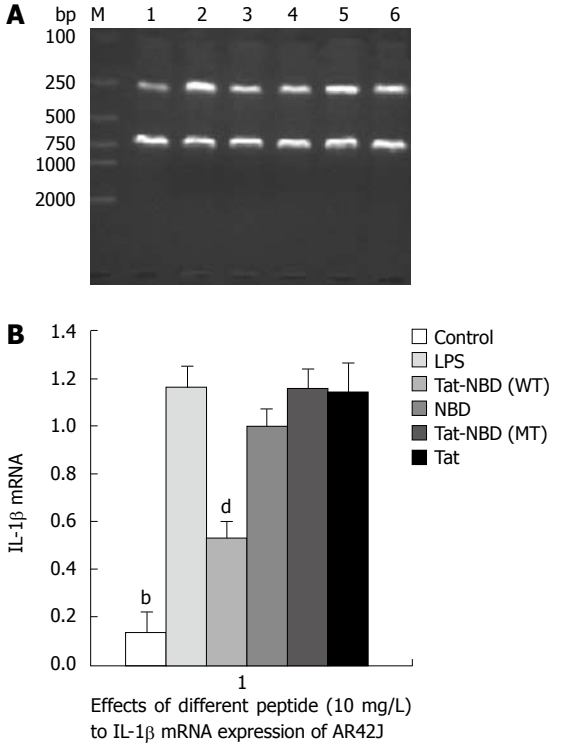

Figure 5 Effects of peptide at a dose of 10 mg/L in *IL-1β* mRNA expression in AR42J by RT-PCR. A: Images of agarose gel electrophoresis; 1: Control; 2: LPS; 3: Tat-NBD (WT); 4: NBD; 5: Tat-NBD (MT); 6: Tat; M: Marker; B: Tat-NBD (WT) peptide decreased *IL-1β* mRNA expression. Control: Cells were incubated with buffer control (n = 3); LPS: Cells were stimulated by LPS for 2 h (n = 3). Tat-NBD (WT): Pretreatment with 10 mg/L of (n = 3); NBD: Pretreatment with 10 mg/L of NBD (n = 3); Tat-NBD (MT): Pretreatment with 10 mg/L of Tat-NBD (MT) (n = 3); Tat: Pretreatment with 100 mg/L of Tat (n = 3). $^{b}P < 0.01$, *vs* LPS group. $^{d}P < 0.01$ *vs* LPS group.

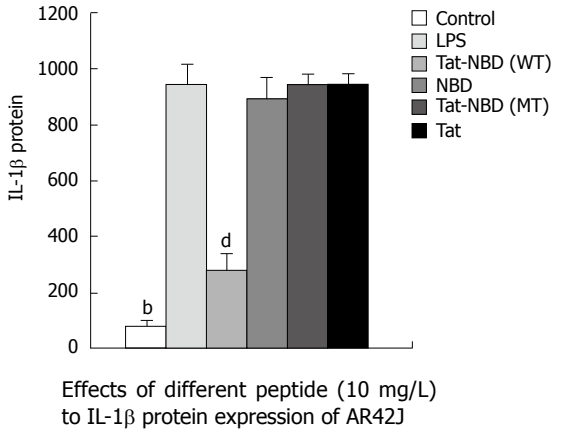

Figure 6 Effects of peptide at dose of 10 mg/L in IL-1β protein expression of AR42J. Control: Cells were incubated with buffer control (n = 3); LPS: Cells were stimulated by LPS for 2 h (n = 3); Tat-NBD (WT): Pretreatment with 10 mg/L of (n = 3); NBD: Pretreatment with 10 mg/L of NBD (n = 3); Tat-NBD (MT): Pretreatment with 10 mg/L of Tat-NBD (MT) (n = 3); Tat: Pretreatment with 100 mg/L of Tat (n = 3). $^{b}P < 0.01$, *vs* LPS group. $^{d}P < 0.01$ *vs* LPS group.

TNF-α: LPS (10 mg/L) resulted in an approximately 10-fold increase of *TNF-α* mRNA, whereas significant inhibitory effects were observed when cells were incubated with Tat-NBD (WT) at a dose of 0.1 mg/L (Figure 7). The data indicate that TAT-NBD (WT)

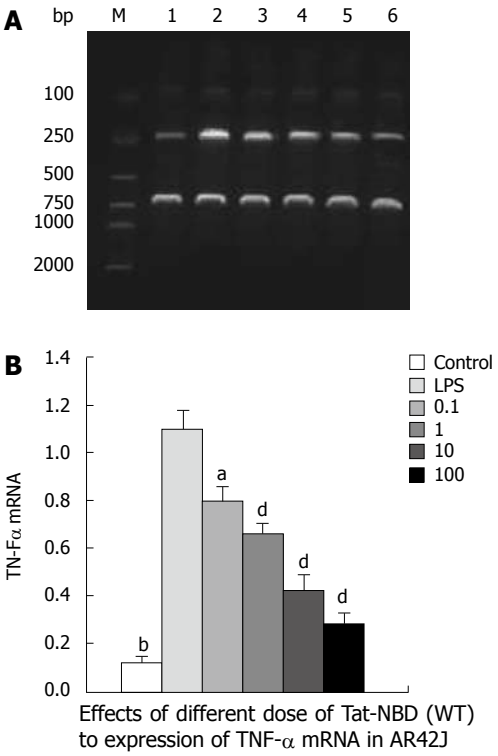

Figure 7 Effects of Tat-NBD (WT) in *TNF-α* mRNA expression of AR42J by RT-PCR. A: Images of agarose gel electrophoresis; 1: Control; 2: LPS; 3: 0.1 mg/L Tat-NBD (WT); 4: 1 mg/L Tat-NBD (WT); 5: 10 mg/L Tat-NBD (WT); 6: 100 mg/L Tat-NBD (WT); M: Marker; B: TAT-NBD (WT) decreased TNF-α protein in a dose-dependent fashion over a range of 0.1 to 100 mg/L. Control: Cells were incubated with buffer control (n = 3); LPS: Cells were stimulated by LPS for 2 h (n = 3). 0.1: Pretreatment with 0.1 mg/L of Tat-NBD (WT) (n = 3); 1: Pretreatment with 1 mg/L of Tat-NBD (WT) (n = 3); 10: Pretreatment with 10 mg/L of Tat-NBD (WT) (n = 3); 100: Pretreatment with 100 mg/L of Tat-NBD (WT) (n = 3). $^aP < 0.05$, $^bP < 0.01$, *vs* LPS group. $^dP < 0.01$ *vs* LPS group.

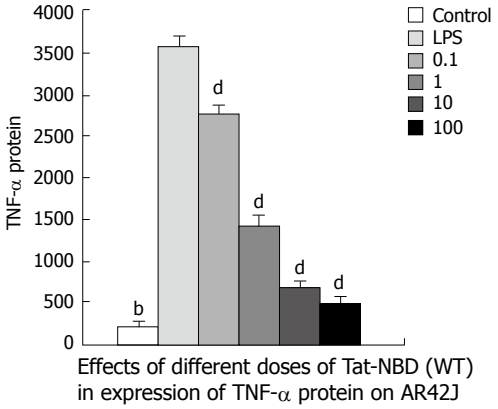

Figure 8 Effects of Tat-NBD (WT) in TNF-α protein expression of AR42J by ELISA. TAT-NBD (WT) decreased TNF-α protein in a dose-dependent fashion over a range of 0.1 to 100 mg/L. Control: Cells were incubated with buffer control (n = 3); LPS: Cells were stimulated by LPS for 2 h (n = 3). 0.1: Pretreatment with 0.1 mg/L of Tat-NBD (WT) (n = 3); 1: Pretreatment with 1 mg/L of Tat-NBD (WT) (n = 3); 10: Pretreatment with 10 mg/L of Tat-NBD (WT) (n = 3); 100: Pretreatment with 100 mg/L of Tat-NBD (WT) (n = 3). $^bP < 0.01$, *vs* LPS group. $^dP < 0.01$ *vs* LPS group.

decreased TNF-α protein in a dose-dependent fashion over a range of 0.1-100 mg/L (Figure 7). We determined whether Tat-NBD (WT) peptide attenuated TNF-α protein, which was activated by LPS. Culture medium

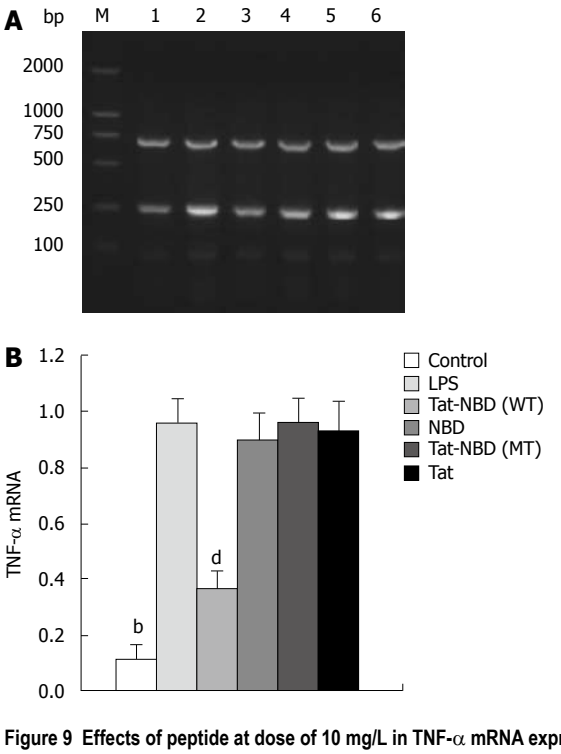

Figure 9 Effects of peptide at dose of 10 mg/L in TNF-α mRNA expression of AR42J by RT-PCR. A: Images of agarose gel electrophoresis; 1: Control; 2: LPS; 3: Tat-NBD (WT); 4: NBD; 5: Tat-NBD (MT); 6: Tat; M: Marker; B: only Tat-NBD (WT) peptide decreased *TNF-α* mRNA expression. Control: Cells were incubated with buffer control (n = 3); LPS: Cells were stimulated by LPS for 2 h (n = 3); Tat-NBD (WT): Pretreatment with 10 mg/L of (n = 3); NBD: Pretreatment with 10 mg/L of NBD (n = 3); Tat-NBD (MT): Pretreatment with 10 mg/L of Tat-NBD (MT) (n = 3); Tat: Pretreatment with 100 mg/L of Tat (n = 3). $^bP < 0.01$ *vs* LPS group. $^dP < 0.01$ *vs* LPS group.

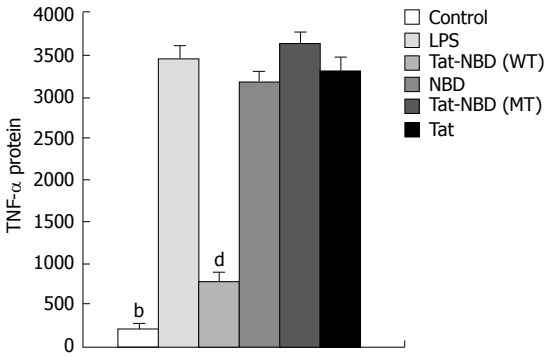

Figure 10 Effects of peptide at dose of 10 mg/L in TNF-α protein expression of AR42J. Control: Cells were incubated with buffer control (n = 3); LPS: Cells were stimulated by LPS for 2 h (n = 3); Tat-NBD (WT): Pretreatment with 10 mg/L of (n = 3); NBD: Pretreatment with 10 mg/L of NBD (n = 3); Tat-NBD (MT): Pretreatment with 10 mg/L of Tat-NBD (MT) (n = 3); Tat: Pretreatment with 100 mg/L of Tat (n = 3). $^bP < 0.01$ *vs* LPS group. $^dP < 0.01$ *vs* LPS group.

was analyzed by ELISA. Result confirmed that TNF-α protein was inhibited by Tat-NBD (WT) at different doses, which also presented in a dose-dependent manner (Figure 8). Finally, we investigated whether the control peptide of NBD, Tat or Tat-NBD (MT) could block LPS-induced TNF-α mRNA or protein increasing in AR42J cells. Cells were preincubated for 2 h at a concentration of 10 mg/L of Tat-NBD (WT) or control prior to stimulation with LPS. Cells were harvested 120 min after addition of LPS. RT-PCR and

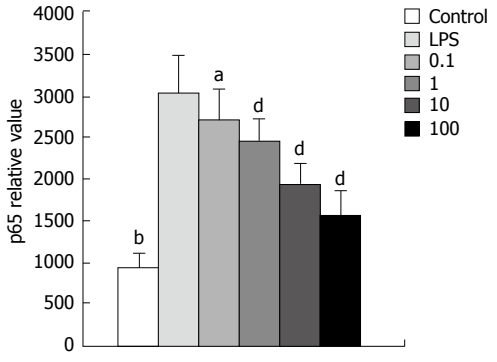

Figure 11 Effects of Tat-NBD (WT) in p65 protein expression of AR42J. Control: Cells were incubated with buffer control (n = 10); LPS: Cells were stimulated by LPS for 2 h (n = 10). 0.1: Pretreatment with 0.1 mg/L of Tat-NBD (WT) (n = 10); 1: Pretreatment with 1 mg/L of Tat-NBD (WT) (n = 10); 10: pretreatment with 10 mg/L of Tat-NBD (WT) (n = 10); 100: Pretreatment with 100 mg/L of Tat-NBD (WT) (n = 10). $^aP < 0.05$, $^bP < 0.01$, *vs* LPS group. $^dP < 0.01$ *vs* LPS group.

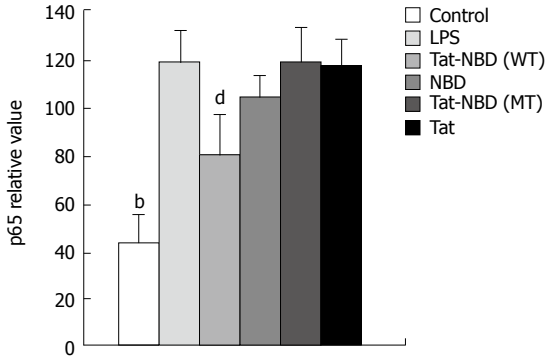

Figure 12 Effects of peptide at dose of 10 mg/L in p65 protein expression of AR42J. Control: Cells were incubated with buffer control (n = 10); LPS: Cells were stimulated by LPS for 2 h (n = 10); Tat-NBD (WT): Pretreatment with 10 mg/L of (n = 10); NBD: Pretreatment with 10 mg/L of NBD (n = 10); Tat-NBD (MT): Pretreatment with 10 mg/L of Tat-NBD (MT) (n = 10); Tat: Pretreatment with 100 mg/L of Tat (n = 10). $^bP < 0.01$ *vs* LPS group. $^dP < 0.01$ *vs* LPS group.

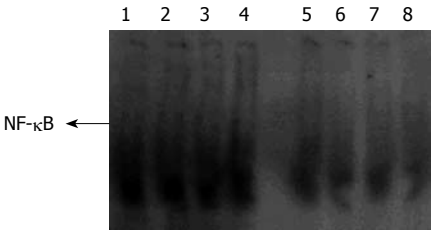

Figure 13 NF-κB activity was obviously activated after LPS induced AR42J (line 1). After cells were pretreated with Tat-NBD (WT), the activity of NF-κB was markedly inhibited in a dose-dependent manner (line 5-8). No change in control peptide group (line 2-3); Lane 1-8: NF-κB activity in LPS group, Tat-NBD (MT) group, Tat group, NBD group, Tat-NBD (WT) group (0.1 mg/L, 1 mg/L, 10 mg/L and 100 mg/L respectively).

ELISA were performed as stated. Only Tat-NBD (WT), not the control peptides, inhibited TNF-α expression (Figures 9 and 10).

NF-κB change in AR42J: To know the role of p65 in the cytokine change in AR42J cells stimulated by LPS and whether the peptide interfered with p65 expression and nucleus transposition, we stained the AR42J cells by IH method. First, we performed experiments to demonstrate that AR42J cells can successfully express p65 under silence condition. Figure 11 shows the increasing histochemical staining of AR42J cells for p65 after stimulation with 0.1 mg/L of LPS. The data indicated that LPS increased p65 in a dose-dependent fashion over a range of 0.1-100 mg/L. We studied the effects of different doses of Tat-NBD (WT) in p65 expression. Cells were preincubated for 2 h at concentrations ranging from 0.1 mg/L to 100 mg/L. After pretreatment of Tat-NBD (WT), weakened immmunocytochemical staining of p65 in AR42J cells was observed, especially in larger dosage. Finally, we investigated whether the control peptide of NBD, Tat or Tat-NBD (MT) could block LPS-induced p65 increasing in AR42J cells. The results showed that only Tat-NBD (WT), not the control peptide inhibited p65 expression. EMSA demonstrated that the NF-κB DNA-binding activity was similar to the IH results (Figures 12 and 13).

DISCUSSION

It is known that a "cascade" induced by recruitment of inflammatory cells and release of inflammatory mediators is an important pathogenesis in the development of acute pancreatitis[17]. Inflammatory mediators, such as LPS and cytokines, may play a critical role in the development of murine acute pancreatitis. NF-κB formed dimeric complex that controls the expression of a variety of inducible genes involved in inflammation and proliferation[13]. It was reported that a selective inhibitor of NF-κB activation, NBD, demonstrated a significant decrease in pancreatic inflammation and lung hemorrhage associated with acute pancreatitis[11]. Here, rats were pretreated with the peptide at a dose larger than that used by Ethridge *et al*[12]. The Tat-NBD or NBD peptide resulted in tissue damage, including edema, hyperemia, necrosis, hemorrhage, and infiltration of inflammatory cells, decreased. Neither mutant Tat-NBD nor Tat peptide had preventative effects. It is suggested that treatment with Tat-NBD or NBD may be effective in this kind of pancreatitis model.

Inflammation and acinar cell death are the hallmarks of both human and experimental pancreatitis[18]. It is not clear whether peptides can have such direct anti-inflammatory effects in acinus cells. LPS stimulates cells by interaction with CD-14 in the context of Toll-like receptor, whose activation leads to NF-κB nuclear translocation through degradation of IκB and subsequent release of NF-κB[19]. The AR42J cell line is the only currently available cell line that maintains many characteristics of normal pancreatic acinar cells, such as the synthesis and secretion of digestive enzymes[20]. AR42J cell receptor expression and signal transduction mechanisms parallel those of pancreatic acinar cells. Thus, this cell line has been widely used as an *in vitro* model to study cellular secretion, growth, proliferation, and apoptosis of the exocrine pancreas[21]. So, we used LPS and AR42J as tools to explore peptide effects

in present study. Further studies showed that in the early stages of LPS-stimulated inflammation in AR42J cells, consisting with sharp increasing expression of cytokine mRNA, such as *IL-1β* and *TNF-α*, which is confirmed as critical factors related to acinus injury[22]. The level of IL-1β and TNF-α protein also were increased in the culture medium. Our data, obtained from parallel studies, demonstrate that pretreatment with different doses of Tat-NBD peptide had constitutive inhibitory effects in acinus cells, resulting in reduced inflammatory cytokines IL-1β and TNF-α. These results are in agreement with previous reports[7,10,11]. We found that the effects of peptide had a dose-dependent manner ranging from 0.1 to 100 mg/L. The largest anti-inflammation effects were found at a dose of 100 mg/L. We used the same concentration of control peptide, NBD, mutate Tat-NBD or Tat, and compared effects with those of Tat-NBD (WT). The results suggest that no control peptide had a preventive role. It has been confirmed that the Tat protein of human immunodeficiency virus 1 (HIV-1) can enter cells efficiently when added exogenously in the tissue culture[23,24]. Also, Tat-mediated uptake may allow the therapeutic delivery of macromolecules previously thought to be impermeable to living cells[23]. Our data show that the sequence of Tat plays an intensive role in NBD sequence, and only the NBD sequence had a therapeutic role in STC-induced rat model as stated previously. The degree of permeability of Tat-NBD and NBD sequences in acinus cells must be confirmed in further studies.

The NBD peptide is a novel six-amino acid cell-permeable peptide that selectively inhibits NF-κB activation *in vitro* and *in vivo* by blocking the interaction of the regulatory protein NEMO with the IKK complex. In the present study, we detected AR42J cell NF-κB activity by EMSA at 24 h after LPS stimulation. During the parallel time course, the DNA-binding of NF-κB markedly inhibited Tat-NBD (WT) peptide (Figure 12), especially at the dose of 100 mg/L. Mutant Tat-NBD, NBD or Tat sequence had no significant effect on NF-κB activity. Consistent with the changes of NF-κB activity, the cell expression of p65 was also attenuated by Tat-NBD (WT) peptide in a dose-dependent manner (Figure 13). Like other studies, our results suggests that the Tat-NBD (WT) peptide plays a part in NF-κB activating pathway.

In conclusion, our results support the idea that active NF-κB participates in the pathogenesis of STC-induced acute pancreatitis in rats. Tat-NBD (WT) peptide has anti-inflammatory effects in this model and inhibits the inflammation of acinus simulated by LPS directly. Inhibition of NF-κB activity may be a protective measure in the treatment of acute pancreatitis.

COMMENTS

Background

The Tat-NEMO-binding domain (NBD) confirmed that it could attenuate the severity of cerulein-induced pancreatitis in mice which improved the inflammation in the pancreas, reduced hemorrhage in the lungs, and lowered myeloperoxidase activity in both pancreas and lung.

Research frontiers

In rat pancreatitis induced by taurocholate, activation of NF-κB in pancreas has been reported. It is not clear yet whether peptide has anti-inflammatory effects in taurocholate-induced severe acute pancreatitis (SAP) model and inhibits the inflammation of acinus stimulated by lipopolysaccharides (LPS).

Innovations and breakthroughs

Tat-NBD peptide is able to inhibit the inflammation of AR42J cells. Further studies are needed to prove its mechanism of action in pancreatic acinar cells.

Applications

Tat-NBD (WT) peptide has anti-inflammatory effects in STC-induced pancreatitis model and inhibits the inflammation of acinus simulated by LPS directly. Inhibition of NF-κB activity may be a protective measure in the treatment of acute pancreatitis.

Peer review

This is an interesting study in which the authors used an alternate approach to demonstrate the influence of inflammatory cytokines involvement in acute pancreatitis.

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ORIGINAL ARTICLES

Astragalus extract inhibits destruction of gastric cancer cells to mesothelial cells by anti-apoptosis

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Abstract

AIM: To determine the inhibitory effect of Astragalus memebranaceushas on gastric cancer cell supernatant-induced apoptosis of human peritoneal mesothelial cells.

METHODS: Human peritoneal mesothelial cell (HPMC) line HMrSV5 was co-incubated with gastric cancer cell supernatant (MKN45) and/or Astragalus memebranaceushas. Morphological changes in gastric cancer cells were observed under phase-contrast microscope. Quantitative cell damage was determined by MTT assay. Apoptosis was determined under transmission electron microscope and quantified by detecting acridine orange/ethidium bromide-stained (AO/EB) condensed nuclei under fluorescent microscope or by flow cytometry. Expressions of Bcl-2 and Bax were evaluated with immunostaining.

RESULTS: Morphological changes and exfoliation occurred and naked areas appeared in cultured HMrSV5 cells 24 h after they were treated with gastric cancer cell supernatant. Cell supernatant from MKN45 gastric cancer cells induced apoptosis of HMrSV5 cells in a time-dependent manner. Obvious morphological changes were observed in cell apoptosis, such as condensation of chromatin, nuclear fragmentations and apoptotic bodies. Astragalus memebranaceus could partly suppress these changes and regulate the expressions of Bcl-2 and Bax in HMrSV5 cells.

CONCLUSION: Gastric cancer cells induce apoptosis of HPMCs through the supernatant. Astragalus memebranaceushas inhibits this phenomenon and can be used an adjuvant chemothera-peutic agent in gastric cancer therapy.

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Key words: Peritoneal carcinomatosis; Stomach neoplasm; Astragalus plant; Mesothelial cell; Apoptosis

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Na D, Liu FN, Miao ZF, Du ZM, Xu HM. Astragalus extract inhibits destruction of gastric cancer cells to mesothelial cells by anti-apoptosis. *World J Gastroenterol* 2009; 15(5): 570-577 Available from: URL: http://www.wjgnet.com/1007-9327/15/570.asp DOI: http://dx.doi.org/10.3748/wjg.15.570

INTRODUCTION

Peritoneal dissemination is one of the most important factors for human gastric cancer and stands in the way of its successful surgical treatment[1]. It appears in the terminal stage and significantly worsens the prognosis of gastric carcinoma. However, the mechanisms underlying this propensity for metastasis are not yet clearly understood.

Peritoneal carcinomatosis can be considered a series of events that form a peritoneal metastatic cascade. Peritoneal stromal tissue appears to be a friendly host for tumor proliferation, providing a rich source of growth factors and chemokines involved in tumor metastasis. At present, our understanding of the molecular mediators that orchestrate this cascade is poor[2]. It has been reported that mesothelial cells prevent cancer invasion and undergo morphologoc changes in response to the factors released by cancer cells[3]. Before invading tumor cells and gaining firm adherence to the submesothelial monolayer, mesothelial cells must penetrate the mesothelial monolayer. Buck *et al*[4] have illustrated the protective effect of mesothelium in a rat model. When Walker 256 tumor cells are injected into the peritoneal cavity of rats, in which the parietal

peritoneum has been stripped of its mesothelial lining leaving the basement membrane intact, rapid implantation occurs in denuded areas[4]. However, in un-operated rats with an intact mesothelium, peritoneal implantation is a rare event. Findings suggest that, prior to gastric cancer cell adhesion to the peritoneum, mesothelial cells become hemispherical, exfoliation occurs, and the naked areas of submesothelial connective tissue are exposed to the peritoneal cavity[5,6]. Invasion of the mesothelial monolayer appears to occur by tumor-induced mesothelial apoptosis[7,8]. In this study, we examine the effects of factors released by gastric cancer cells on peritoneal mesothelial cell apoptosis *in vitro*.

Astragalus memebranaceus is a traditional Chinese herbal medicine used in treatment of common cold, diarrhea, fatigue, anorexia and cardiac diseases[9-11]. It also has been used as an immunomodulating agent in treatment of immunodeficiency diseases, to alleviate the adverse effects of chemotherapeutic drugs[12-14], such as significantly reduced myelosuppression in cancer patients[15,16]. The active pharmacological constituents of radix Astragalus memebranaceus include various polysaccharides, saponins and flavonoida as well as L-arginine or L-canavanine[17]. Moreover, studies also showed that Astragalus polysaccharides have effects on colon cancer, mammary tumor, urological tumor, gastric cancer, *etc.*[18,19]. In recent years, it has been proposed that Astragalus may possess anti-apoptosis potential in peritoneal mesothelial cells[20,21]. In spite of this, the anti-apoptotic effects of Astragalus saponin extract on human peritoneal mesothelial cells during peritoneal carcinomatosishas have not been extensively studied. This study was to observe the anti-apoptosis effects of Astragalus saponin extract on human peritoneal meso-thelial cells during peritoneal gastric cancer metastasis.

MATERIALS AND METHODS

Reagents

Astragalus injection was obtained from Chiatai Qingchunbao Pharmaceutical Co. Ltd (China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide (MTT) and acridine orange/ethidium bromide (AO/EB) were obtained from Fluka (USA). Propidium iodide (PI) was obtained from Biosharp (USA). Bcl-2, Bax and actin primary antibodies, as well as second antibodies goat anti-mouse IgG were obtained from Santa Cruz Biotechnology Inc (CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from GIBCOBRL (Grand Island, NY, USA). Other laboratory reagents were obtained from Sigma (USA). Phase-contrast microscope (Japan, Nikon), fluorescence microscopy (Japan, Olympus), transmission electron microscopy (Japan, Hitachi H-6001), flow cytometry (FACScalibur; Becton Dickson, USA), were also used in this study.

Cell line and cell culture

Human peritoneal mesothelial cell line HMrSV5 was kindly provided by Professor You-Ming Peng, Second Hospital of Zhongnan University (Changsha, China) and Professor Pierre RONCO, Hospital TENON (Paris, France). Human gastric carcinoma metastatic liver cell line, MKN-45, and normal gastric epithelial cell line, GES-1, were obtained from Beijing Cancer Research Institute, China. The cells were placed into 75 cm^2 tissue culture flasks and grown at 37℃, under a humidified atmosphere containing 50 mL/L CO_2 and 950 mL/L air, in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin, 2 mmol/L L-glutamine, 20 mmol/L hydroxyethyl piperazine ethanesulfonic acid (HEPES). The medium was changed every two or three days. The cells were dislodged using 0.25% trypsin and 0.02mol/L EDTA in PBS for subculture.

Preparation of serum-free conditioned media

Serum-free conditioned media (SF-CM) from gastric cancer cells or normal gastric epithelial cells were prepared as previously described[1]. Briefly, 5.0×10^5 cells were seeded into 100 mm tissue culture dishes with 10 mL DMEM supplemented with 10% FCS and incubated at 37℃ for 3 d. To obtain SF-CM, the cells were washed twice with PBS and then incubated for 2 d with 3 mL of serum-free DMEM. SF-CM were eluted and centrifuged at 1000 × *g* for 5 min, passed through filters (pore size, 0.45 μm) and stored at -20℃ until use.

Human peritoneal mesothelial cell line HMrSV5 was cultured to sub-confluence in a 50 cm^2 dish with 10% FCS containing DMEM. The medium was changed to 4 test solutions: (1) serum free DMEM, (2) SF-CM from gastric cancer cell line MKN45, (3) SF-CM from gastric cancer cell MKN45 + 200 μg/mL Astragalus injection, (4) DMEM + 200 μg/mL Astragalus injection.

Morphologic evaluation under a phase-contrast microscope

Mesothelial cells in 24-well chambers were exposed to test solutions for 24 h, gently washed with PBS, and then examined under a phase-contrast microscope for the size, shape, and integrity of cell membrane, cytoplasm, and nuclei.

Transmission electron microscopy

After incubation in test solutions for 24 h, the cells were trypsinized and then fixed in PBS (pH 7.3) containing ice-cold 2.5% glutaraldehyde. The specimens were rinsed with PBS, post-fixed in 1% osmium tetroxide containing 0.1% potassium ferricyanide, dehydrated through a graded series of ethanol (30%-90%), and embedded in Epon. The specimens were cut into semi-thin (300 nm) sections with a Reichart ultra-cut, stained with 0.5% toluidine blue, and examined under a light microscope. The ultra-thin sections (65 nm) were stained with 2% uranyl acetate and Reynold's lead citrate, and examined under a transmission electron microscope (× 5000 or × 8000 magnification).

Quantitative determination of cell damage by MTT assay

Tests were performed to assess mesothelial cell viability

after treatment with SF-CM for gastric cancer cell MKN45 and Astragalus injection. After exposure to the control or test solutions for a specific period (observed at 12 h, 24 h, 48 h), the cells in 96 well plates were incubated with 50 μg/mL MTT at a dilution of 1:10 based on the volume of culture medium, for 3 h at 37℃. At the end of incubation, MTT solution was removed, 150 μL DMSO was added to each well and stirred to dissolve the dark-blue formazon crystals formed. The proportion of viable cells was determined by measuring the optical density of each sample at 480 nm with a spectrophotometer. The cells were exposed three times to each solution for the same period of time. The means for the groups of cultures were compared. Control wells with no Astragalus injection and SF-CM were added. The same test was performed to assess the MKN45 viability after treatment with Astragalus injection.

Flow cytometry

After incubation in test solutions for 12, 24 and 48 h, the cells were harvested by trypsinization, resuspended in PBS at a concentration of 1×10^6/mL and fixed in 2 mL methanol for 30 min at 4℃. After mesothelial cells were fixed, the mixture was incubated in 0.5 mL propid iumiodide (PI) solution (0.05 mg/mL in 3.8 mol/L Na citrate) and 0.5 mL RNAse A (0.5 mg/mL) at room temperature for 30 min. Finally, the cells were resuspended in 1 mL PBS and analyzed by flow cytometry according to the manufacturer's instructions. The cells in the subdiploid peak were considered apoptotic.

In situ detection of apoptosis

Human peritoneal mesothelial cells were cultured to sub-confluence in a 24-chamber slide with 10% FCS containing DMEM. The medium was changed to (1) serum free DMEM, (2) SF-CM from gastric cancer cell MKN45, (3) SF-CM from MKN45 + 200 μg/mL Astragalus injection, (4) DMEM + 200 μg/mL Astragalus injection, and (5) SF-CM from normal gastric epithelial cell line GES-1, respectively. After incubation for 48 h, apoptosis was quantified with fluorescent staining method by detecting AO/EB condensed nuclei with fluorescent microscopy. AO/EB staining could identify alive, early and late apoptotic cells and necrotic cells. Sub-confluent cells (70%-80% confluent) in 24 well uncoated plates were exposed to apoptotic stimuli for 48 h. Mesothelial cells in 24 well plates were gently washed with PBS and immediately treated with acridine orange (100 μg/mL) for 5 min and ethidium bromide (100 μg/mL) for 5 min. Each well was then examined under an epifluorescence microscope. An excitation wavelength of 455 nm was used to evaluate apoptosis under a fluorescence microscope. Apoptosis was defined by morphological criteria. Cells containing normal nuclear chromatin exhibited green nuclear staining. Cells containing fragmented nuclear chromatin exhibited orange to red nuclear staining.

Western blot

Human peritoneal mesothelial cells were cultured to sub-confluence in a 50 cm^2 dish with 10% FCS containing DMEM. The media were then changed to test solutions for 24 h. The cells were lysed in an ice-cold lysing solution containing 154 mmol/L NaCl, 1 mmol/L EDTA, 1% octylphenoxy polyethoxyethanol, 1 mmol/L phenylmethanesulfonyl fluoride, 1 mg/mL pepstatin, 1 mg/mL aprotinin, and 0.25% nadeoxycholate. Samples were rotated for 15 min at 4℃ and then centrifuged at 12000 r/min for 5 min at 4℃. The supernatant was recovered, and protein concentration was measured by bicinchoninic acid assay (Bio-Rad), with bovine serum albumin as the standard. Samples were incubated for 5 min at 95℃ in a loading buffer (12 mmol/L Tris-HCl, pH 6.8, with 25% glycerol, 2% sodium dodecyl sulfate, 14.4 mmol/L 2-mercaptoethanol, and 0.1% bromophenol blue), and 50 mg of protein was loaded on different percentages different percentages of SDS-polyacrylamide gels (exclusion limits) corresponding to the molecular weight of target proteins. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane by electro-blotting. The membrane was blocked in 1% BSA, 0.05%Tween and PBS solution overnight at 4℃. Mouse antihuman monoclonal antibodies to Bcl-2 and Bax were used as primary antibodies. Horseradish peroxidase-labelled goat anti-mouse IgG was used as a secondary antibody. Blots were developed by incubation in a chemiluminescence substrate and exposed to X-ray films.

Statistical analysis

All data were expressed as mean ± SE. Student's *t*-test was used to compare drug effects. $P < 0.05$ was considered statistically significant.

RESULTS

Morphologic evaluation under a phase-contrast microscope

A typical polygonal cobblestone was found in untreated mesothelial cells in SF-CM (Figure 1A), but morphological changes occurred in treated mesothelial cells in SF-CM. The most obvious morphological changes in mesothelial cells was exfoliation and naked areas (Figure 1B). Astragalus injection could partly suppress such morphological changes (Figure 1C).

Transmission electron microscopy

Twenty-four hours after treatment of MKN45, the apoptotic features (such as condensation of nuclear chromatin, wrinkling of nuclear membrane, dilation of endoplasmic reticulum, and relatively normal structure of mitochondria) were verified by electron microscopy (Figure 2). Under transmission electron microscope, the membrane of nuclei was complete, and the chromatin concentrated into masses on the boundary of the membrane or formation of arch (Figure 2C). Phenomena of budding and formation of apoptotic bodies were also observed (Figure 2D).

MTT assay

Anti-apoptotic effects of Astragalus injection were observed in SF-CM and apoptosis of gastric cancer cells

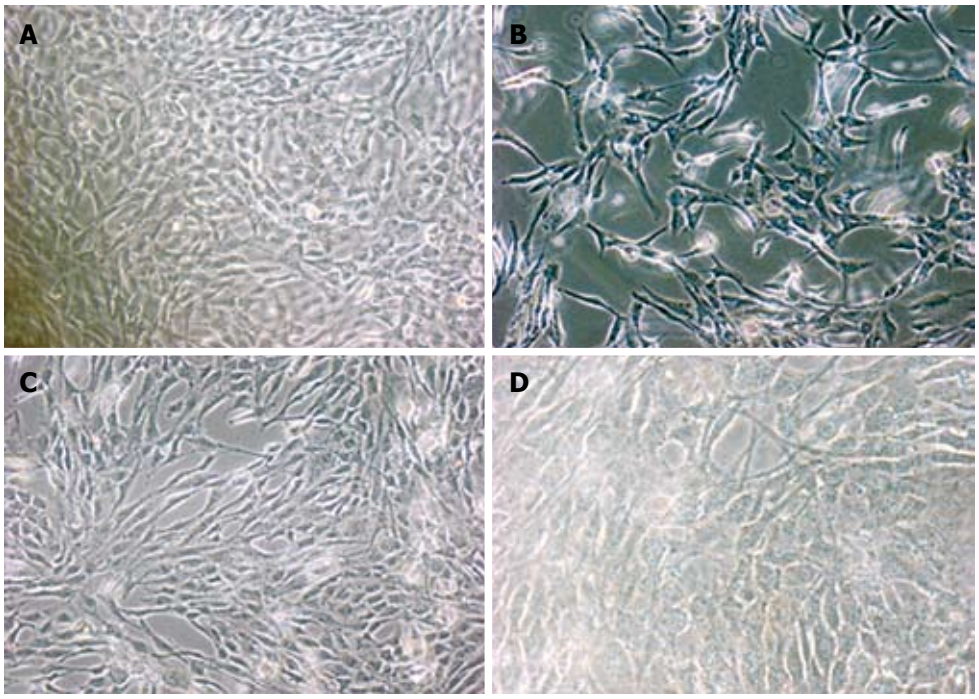

Figure 1 Morphological changes in human peritoneal mesothelial cells under phase contrast microscopy (× 40). A: Morphology of mesothelial cells cultured in serum free DMEM; B: Exfoliation and naked areas of mesothelial cells after treatment with Astragalus injection; C: Morphological changes in mesothelial cells after treatment with 200 μg/mL Astragalus injection; D: No typical morphological changes in mesothelial cells after treatment with DMEM + 200 μg/mL Astragalus injection.

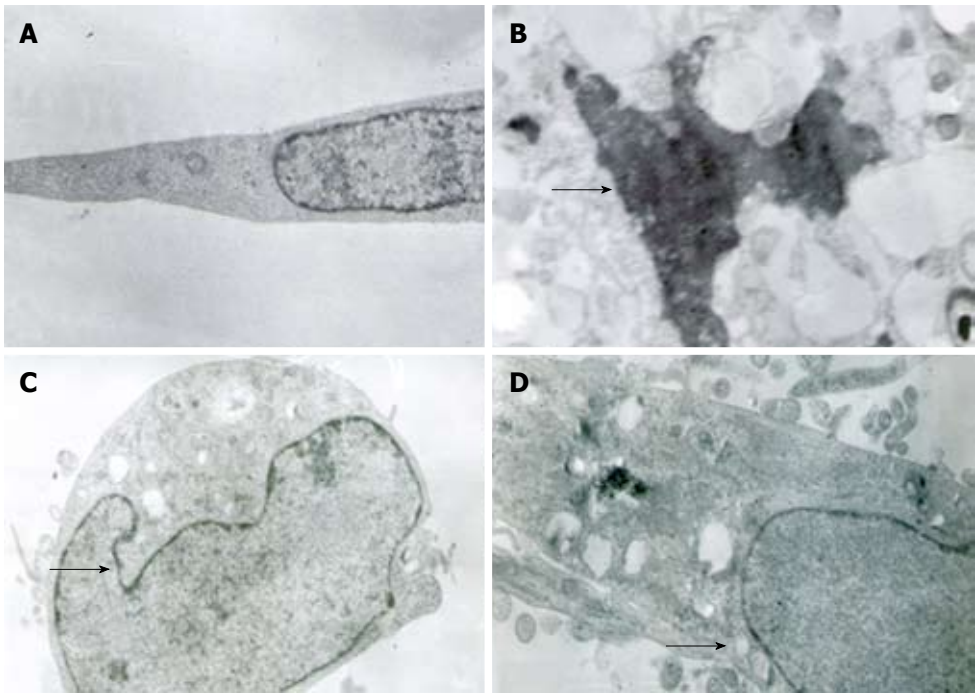

Figure 2 Mesothelial cells under electron microscope after incubation with and without SF-CM. A: Normal nuclei and endoplasmic reticulum of control cells; B: Condensation of nuclear chromatin (arrow in Figure 2B); C: Wrinkling of nuclear membrane of cells after treatment with Astragalus injection (arrow in Figure 2C); D: Dilated endoplasmic reticulum (arrow in Figure 2D).

shared the common intracellular signaling pathways. To evaluate the potential anti-apoptosis effects of combined SF-CM and Astragalus injection on mesothelial cells, we examined the cytotoxic effect of SF-CM and Astragalus injection on human mesothelial cell line HMrSV5. SF-CM could effectively inducing apoptosis of mesothelial cells in a time-dependent manner (Figure 3). Astragalus injection could effectively suppress mesothelial cell apoptosis in SF-CM at 0, 12, 24 and 48 h, respectively.

To evaluate the potential effects of Astragalus injection on MKN45 cells, we examined the cytotoxic effect of Astragalus injection on MKN45 cells. No significant effect of Astragalus injection on MKN45 cell viability was observed ($P = 0.995$, Table 1).

Flow cytometry assay (FCA)

After incubation for 12 h, flow cytometry showed that the apoptosis peak occurred ahead the diploid peak when mesothelial cells were incubated with SF-CM for MKN45 (9.51% ± 0.71%). In contrast, the apoptosis peak value was not increased in the serum free DMEM group (0.70% ± 0.16%). The apoptosis peak value of was 1.65% ± 0.09% in the serum free DMEM group after treatment with Astragalus injection. The percentage of apoptosis was less than 0.5% (0.3% ± 0.07%) in the serum free DMEM group after treatment with DMEM + 200 μg/mL Astragalus injection. The data demonstrate that Astragalus injection could augment anti-apoptosis effects on MKN45 cells (Figure 4) and could effectively

Table 1 MKN45 viability after treatment with 200 μg/mL Astragalus injection

Group	Optical density
Control (MKN45)	2.096 ± 0.834
MKN45 + Astragalus 200 μg/mL	2.183 ± 0.844

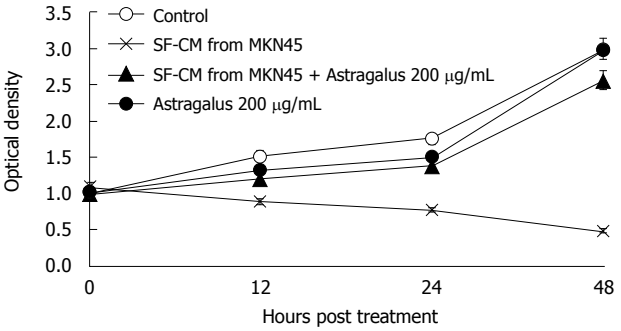

Figure 3 Viability of mesothelial cells after treatment with SF-CM and Astragalus injection. Mesothelial cells (5×10^3 cells/well) were treated with SF-CM, 200 μg Astragalus injection, and SF-CM plus 200 μg Astragalus injection for 12, 24 and 48 h, respectively. Mesothelial cells cultured in serum free DMEM served as controls. MTT assays were performed in triplicate for each data point.

suppress mesothelial cell apoptosis induced by MKN45 as detected by MTT (Figure 5).

In situ detection of apoptosis

After mesothelial cells were treated with SF-CM from gastric cancer cell line MKN45 for 48 h, marked morphological changes in cell apoptosis occurred, such as condensation of chromatin, nuclear fragmentation and apoptotic bodies (Figure 6). Compared with the other three groups, the number of apoptotic cells significantly increased after treatment with SF-CM from gastric cancer cell line MKN45.

The number of apoptotic cells decreased significantly, while the number of green cells increased (Figure 6B and C). Astragalus injection inhibited the apoptosis of mesothelial cells induced by gastric cancer cells, while normal gastric epithelial cell line GES-1 could not induce apoptosis of mesothelial cells (Figure 6E). The number of apoptotic cells decreased after treatment with Astragalus injection (Figure 6B and C).

Western blot

We further delineated the underlying mechanisms of Astragalus injection by which apoptosis of mesothelial cell apoptosis is induced. We examined the effects of Astragalus injection on the expression of Bcl-2 and Bax. Bcl-2 expression was increased and Bax expression was reduced in mesothelial cells 24 h after treatment with Astragalus injection (Figure 7).

DISCUSSION

It has been reported that mseothelial cells can prevent cancer invasion and undergo morphologic changes

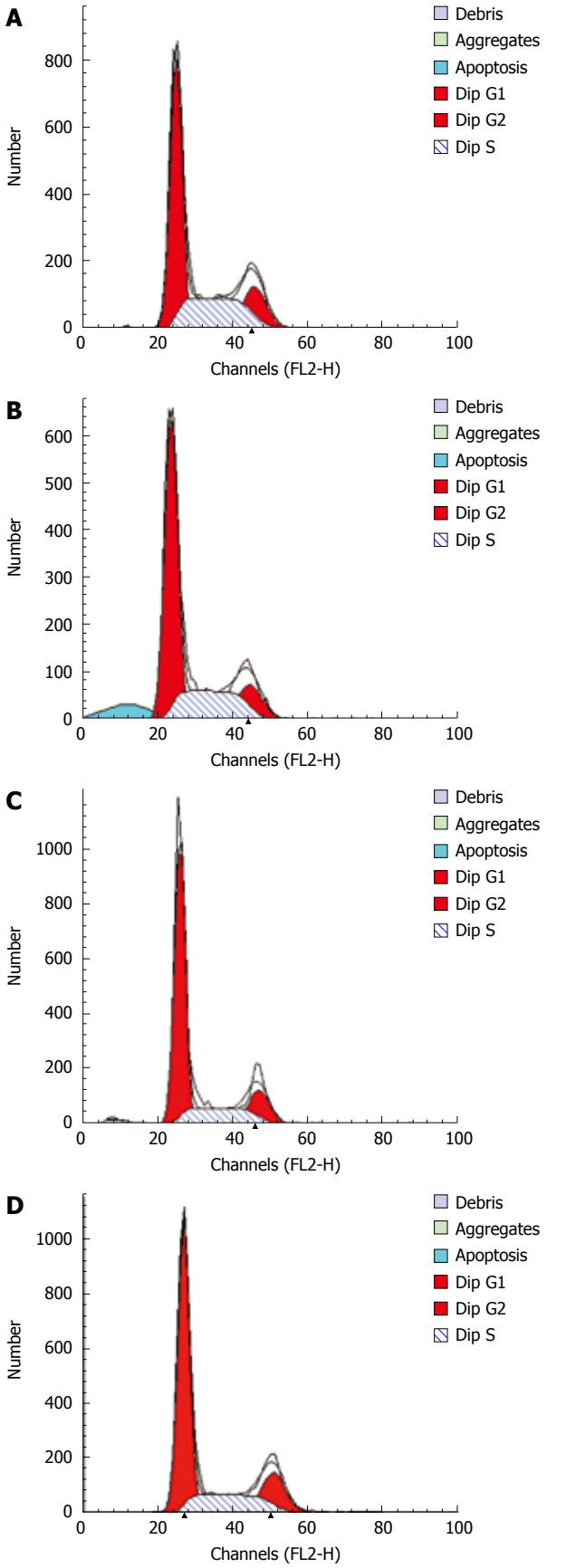

Figure 4 Effect of MKN45 and/or Astragalus injection on cell-cycle distribution in mesothelial cells treated for 12 h. DMEM with an apoptotic rate of 0.52% (A), SF-CM with an apoptotic rate of 10.03% (B), SF-CM plus 200 μg Astragalus injection with an apoptotic rate of 1.57% (C), DMEM + 200 μg/mL Astragalus injection with an apoptotic rate of 0.25% (D). Samples were analyzed by flow cytometry as described in "Materials and methods" section. Apoptotic peak is shown in green.

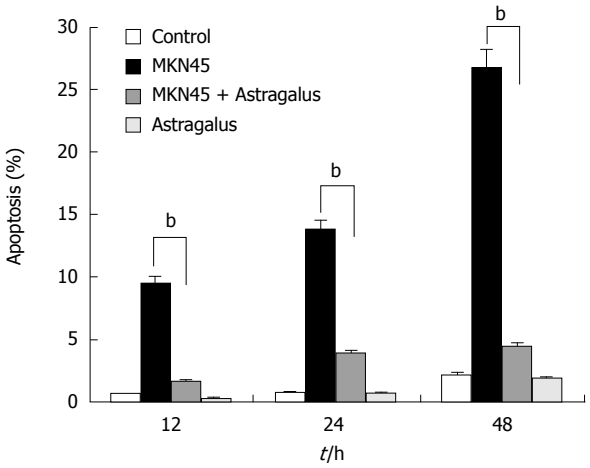

Figure 5 Percentages of mesothelial cells in sub-G1 group (apoptosis) after treatment with control, MKN45, MKN45 + 200 μg/mL Astragalus injection and 200 μg/mL Astragalus injection for various periods of time. $^{b}P < 0.01$ *vs* 12 and 24 h.

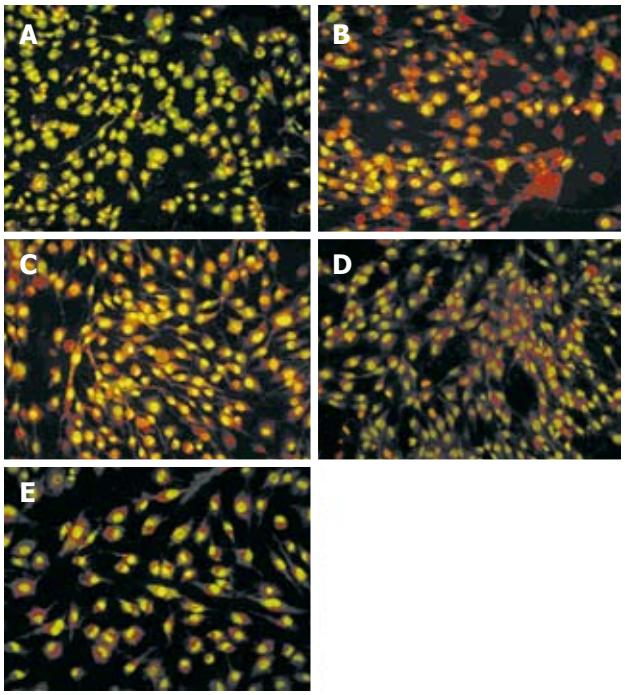

Figure 6 Apoptosis of mesothelial cells treated for 48 h. A: serum free DMEM; B: MKN45; C: MKN45 + 200 μg/mL Astragalus injection; D: DMEM + 200 μg/mL Astragalus injection; E: GES-1 with AO/EB staining. Cells containing normal nuclear chromatin exhibit green nuclear staining. Cells containing fragmented nuclear chromatin exhibit orange to red nuclear staining.

in response to factors released by cancer cells[22]. This phenomenon can be explained by the "seed and soil" theory: metastases occur when some tumor cells only live and grow in a congenial environment. Peritoneum might be a congenial environment for scirrhoid gastric cancer cells. Mesothelial cells can prevent cancer cell infiltration into sub-mesothelial connective tissue. It was reported that abdominal cavity cancer cells release early inflammatory factors and induce apoptosis of peritoneal mesothelial cells[23,24]. When mesothelial cells

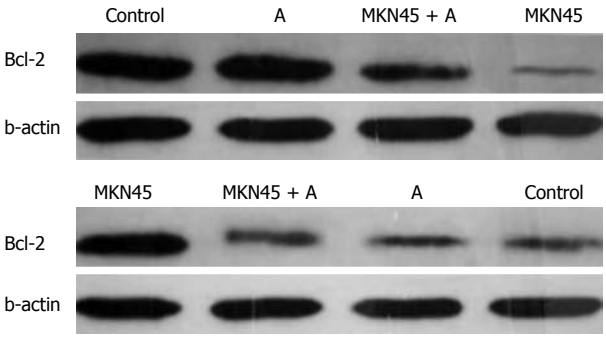

Figure 7 Western blotting analyses of Bcl-2 and Bax proteins. Mesothelial cells were treated for 24 h with serum free DMEM, MKN45, MKN45 + 200 μg/mL Astragalus injection (MKN45 + A), DMEM + 200 μg/mL Astragalus injection, respectively.

become hemispherical, exfoliation occurs, and naked areas of sub-mesothelial connective tissue are exposed to the peritoneal cavity, and this injured peritoneum is a congenial environment for peritoneal metastasis[25].

In the present study, gastric cancer cell supernatant could significantly affect mesothelial cell viability, whereas normal gastric epithelial cell line GES-1 could not. Under contrast phase microscopy, mesothelial cells became hemispherical and exfoliation occurred when serum-free conditioned media were added into cultured mesothelial cells. Furthermore, cytoplasmic atrophy, nuclear shrinkage, and formation of extracellular and/or intracellular apoptotic bodies were observed under a transmission electron microscope. Astragalus injection could partly suppress these morphological changes. Moreover, apoptosis was also quantified by fluorescence microscopy and flow cytometry, showing that factors released by gastric cancer cells in the abdominal cavity induce apoptosis of mesothelial cells, cause exfoliation, and eventually result in metastasis. These may be the mechanisms by which cancer cells adhere to sub-mesothelial connective tissue. More tests will be conducted to better characterize the apoptotic signaling induced by gastric cancer cells.

Our study demonstrated that Astragalus injection could inhibit apoptosis of human peritoneal mesothelial cells induced by gastric cancer cell supernatant, suggesting that morphological changes in human peritoneal mesothelial cells are significantly decreased when human peritoneal mesothelial cells are exposed to both Astragalus injection and gastric cancer cells. Moreover, Astragalus injection could regulate the expression of Bcl-2 and Bax.

Bcl-2, an inhibitor of the mitochondrial apoptosis pathway, exerts its action by blocking proapoptotic counterparts, which in turn prevents release of cytochrome C and activation of caspases[26,27]. The results of this study show that treatment of mesothelial cells with Astragalus injection significantly increased Bcl-2 expression in mesothelial cells, indicating that Astragalus injection exerts its antiapoptotic effects by maintaining the expression of Bcl-2 (Figure 6).

Bax is a death promoter, which is neutralized by heterodimerization with Bcl-2. When Bax translocates

into the outer mitochondrial membrane, a leakage of cytochrome C from the mitochondria into the cytosol occurs[28]. Caspases-9 and -3 are activated sequentially, leading to breakdown of chromosomal DNA. In the present study, MKN45 significantly increased the Bax expression in mesothelial cells, while Astragalus injection partly suppressed the expression of Bax, indicating that there is a great possibility that Astragalus injection-mediated antiapoptosis of mesothelial cells is due to the regulation of Bcl-2 and Bax activation. Hence, identification of target compounds is imminent.

In general, investigation of the application of Astragalus injection in protecting human mesothelial cells is lacking. Astragalus injection has only been used in peritoneal dialysis therapy[29]. Studies on Astragalus injection were mainly concentrated on its immunomodulating functions. Recently, Astragalus injection has been shown to have anticancer activity. In this study, we demonstrated the apoptotic effects of gastric cancer cell supernatant on human peritoneal mesothelial cells during peritoneal gastric cancer metastasis.

In conclusion, Astragalus injection can suppress apoptosis of mesothelial cells and can thus be used in treatment of gastric cancer.

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The authors thank Professor Feng Li for technical assistance and Yan Song, MD, Dr. Hui Gu, and Dr. Qiang Ke for their precious advices.

COMMENTS

Background

Peritoneal carcinomatosis is a common form of disease progression in gastrointestinal cancer. Gastric cancer-induced apoptosis of peritoneal mesothelial cells has been assumed to be an important part in gastric cancer peritoneal metastatic cascade. Astragalus extract, an active component isolated from Astragalus Root, has recently been reported to have anti-apoptosis activities.

Research frontiers

In general, investigations on the application of Astragalus injection in protecting human mesothelial cells are lack. Astragalus injection has only been used in peritoneal dialysis therapy. Studies on Astragalus injection were mainly concentrated on its immunomodulating functions. Recently, Astragalus injection has been shown to have anticancer activity. The objective of this study was to determine the inhibitory effect of Astragalus memebranaceushas on apoptosis of human peritoneal mesothelial cells induced by gastric cancer cell supernatant.

Innovation and breakthroughs

The inhibitory effects of Astragalus injection on apoptosis of human peritoneal mesothelial cells during peritoneal gastric cancer metastasis were investigated in this study.

Applications

Astragalus injection could suppress apoptosis of mesothelial cells and can thus be used in treatment of gastric cancer.

Terminology

Human peritoneal mesothelial cells (HPMC) are sentinel cells that can sense and respond to signals within their microenvironment. Calycosin is a vasorelaxant with no competitive Ca^{2+} release.

Peer review

This is a very excellent work. The authors demonstrated the anti-apoptosis effects of Astragalus on human peritoneal mesothelial cells during peritoneal gastric cancer metastasis.

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BRIEF ARTICLES

Hepatocellular carcinoma in patients with autoimmune hepatitis

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Abstract

AIM: To evaluate and confirm the low incidence of hepatocellular carcinoma (HCC) in patients with autoimmune hepatitis (AIH). At present only very few cases of HCC in patients with AIH and definite exclusion of chronic viral hepatitis have been published, suggesting that HCC due to AIH is rare.

METHODS: In order to further investigate the incidence of HCC in patients with AIH, we reviewed our large cohort of 278 patients with AIH.

RESULTS: Eighty-nine patients (32%) were diagnosed with liver cirrhosis, a preneoplastic condition for HCC. We studied a total of 431 patient years of cirrhosis in these patients, an average 4.8 years per patient. During this period none of the patients of our own study cohort developed HCC. However, three patients with HCC due to AIH associated liver cirrhosis were referred to our department for further treatment of HCC. In all three patients chronic viral hepatitis was excluded.

CONCLUSION: We conclude that HCC may under rare circumstances develop due to chronic AIH dependent liver cirrhosis. Compared to other causes of liver cirrhosis such as chronic viral hepatitis, alcohol, or hemochromatosis, the incidence of HCC is significantly lower. Pathophysiological differences between AIH and chronic viral hepatitis responsible for differences in the incidence of HCC are yet to be further characterized and may lead to new therapeutic concepts in prevention and treatment of liver cancer.

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Key words: Autoimmune hepatitis; Hepatocellular carcinoma; Hepatic C virus; Hepatic B virus; Liver cirrhosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is among the most common malignancies world wide and its incidence is rising, especially in Asia and Sub-Saharan Africa, but also in Western countries[1,2]. Most HCCs develop on the basis of liver cirrhosis due to chronic liver disease, making it a prerequisite for development of the disease. HCCs in non-cirrhotic livers are rare and mostly due to aflatoxin B or hepatitis B. Although HCC may develop as a consequence of all entities of underlying liver disease, it has been noticed before that the incidence of primary liver cancer varies significantly between different forms of chronic liver disease leading to cirrhosis and subsequent liver cancer. In hepatitis C patients suffering from liver cirrhosis, the average yearly incidence of HCC has been published as being approximately 1%-4% in Western countries[3-6]. An even higher rate of up to 8% was reported in Asia[1]. Similarly, the corresponding cumulative incidence rates of HCC in patients with chronic

viral hepatitis B were 1.3% and 14.9% per person-year, respectively, depending on HBV DNA levels[7,8]. The risk for HCC in decompensated alcohol-induced cirrhosis approaches 1% per year[9] and was demonstrated to be less in patients without co-infection of chronic viral hepatitis. Furthermore, the rate for HCC developing on the basis of hemochromatosis was reported between 2% and 6% per patient year[10,11]. Finally, HCC due to late stage primary biliary cirrhosis (PBC) was also reported as up to 2% per patient year in late stage (stage IV) PBC[12,13].

For patients with liver cirrhosis due to autoimmune hepatitis (AIH), only very few patients with HCC have been reported, mostly as single case reports. In addition, since in most earlier studies hepatic C virus (HCV) co-infection was not excluded, only very few data exist on the incidence and occurrence of HCC solely on the basis of AIH and subsequent liver cirrhosis[14,15] (Table 1).

However, confirmation of a low incidence of HCC in patients with AIH would point towards a (patho-) physiological mechanism in patients, preventing a much higher incidence as compared to patients with chronic viral hepatitis C. Further characterization of this phenomenon and patient collectives with HCC due to AIH may subsequently lead to identification of protective mechanisms against liver cancer and open new therapeutic perspectives.

Table 1 Summary of the incidence of HCC in patients with liver cirrhosis due to different underlying diseases. Incidence of HCC was demonstrated to be by far the lowest in autoimmune hepatitis

Patients with liver cirrhosis		
Underlying disease	**HCC incidence per year (%)**	**Reference**
Chronic hepatitis C	1-8	[1,3-6]
Chronic hepatitis B	1-15	[7,8]
Alcoholic liver disease	1	[9]
Hemochromatosis	2-6	[10]
Primary biliary cirrhosis (PBC)	2	[12,13]
Autoimmune hepatitis	< 0.2	

MATERIALS AND METHODS

Study cohort

Two hundred seventy-eight patients satisfied the international criteria for the diagnosis of AIH[16]. They had been enrolled in our chronic liver disease program from 1970 until present and were reviewed for evidence of HCC. All patients had been followed in a uniform fashion for a total of 1951 years (0-34 years). On the basis of immunoserological assessment for autoantibodies, 207 patients were assessable for subtypes of AIH. Of these 195 patients (94%) were classified as type-1 AIH due to positive ANA, SMA, or anti-SLA/LP autoantibodies[17,18]. Eleven (6%) patients had elevated LKM autoantibodies, characteristic for type Ⅱ AIH[19]. Seventy-two patients had no detectable autoantibodies. Follow-up was usually maintained at three-month or six-month intervals during treatment discontinuation and for at least one year after treatment. Thereafter, follow-up assessments were performed at annual intervals, if the clinical condition was stable. Hepatic ultrasound and serum α-fetoprotein determinations were usually repeated every 6-12 mo. Follow up on patients with diagnosed liver cirrhosis was documented for 431 patient years. Follow up on patients not suffering from liver cirrhosis accumulated to 1183 patient years. The diagnosis of HCC required histological documentation.

Immunoserological assessments

SMA, ANA, and LKM were analyzed by indirect immunofluorescence on murine tissue sections as described previously. Anti-SLA/LP were analyzed by inhibition ELISA[18]. A serum titer of 1:80 or higher was considered positive for ANA[17]. A titer of 1:40 or higher was considered positive for SMA. A titer of 1:40 or higher was considered positive for anti-LKM-1[19]. All patients were tested for SMA and ANA, and 193 patients (91%) were tested for anti-LKM-1. Patients with SMA and/or ANA in the absence of anti-LKM-1 were classified as type-1 AIH. Patients with anti-LKM1 were classified as type-2 AIH[19].

Virological assessments

Antibodies to anti-HCV were detected by a second-generation enzyme linked immunosorbent assay (ELISA) and HCV RNA was assessed in serum by PCR. Hepatitis B surface antigen (HBsAg) was determined by ELISA.

HCC patient 1

A 59-year-old male presented at our institution with hydrops decompensated cirrhosis (Child-Pugh Score C) and suspected HCC due to previously diagnosed AIH (SMA positive, anti-SLA/LP positive) and overlap syndrome to primary biliary cirrhosis. The patient had previously been treated at an external hospital over 25 years for cryptogenic hepatitis. Through the course of disease, the patient had been intermittently treated with prednisolone, and at presentation at our department treatment consisted of prednisolone 15 mg/d. For 5 years prior to his presentation at our department, the patient had intermittently been treated with lactulose, pointing to presence of decompensated liver cirrhosis and thus, retrospectively, liver cirrhosis must be assumed to have existed several years prior to presentation. Esophageal varices were known at least 2 years prior to presentation at our department. An accompanying chronic viral hepatitis was excluded by means of serological testing.

After presentation at our department a large liver tumor measuring 9.8 cm × 8 cm × 7.5 cm was found by ultrasound and CT scan. Histology confirmed the diagnosis of HCC. Due to the lack of surgical options, the patient underwent transarterial chemoembolization as palliative treatment. Initially, the intervention was performed without complications and the patient was released from the hospital in good condition. However, three weeks later, the patient was re-admitted to our emergency department with abdominal pain and signs

of subileus. Splenic rupture was identified. The patient died the following day.

HCC patient 2

A 78-year-old female was admitted to our hospital with a confirmed longstanding course of AIH under immunosuppressive therapy. ANA and SMA autoantibodies were positive with high titers. Liver cirrhosis (Child-Pugh-Score A) had previously been diagnosed. Initial diagnosis of AIH was made 12 years prior to presentation at our department and the patient was treated with an immunosuppressive regimen. At presentation at our department medication consisted of prednisolone 2.5 mg/d and azathioprine 100 mg/d. An accompanying chronic viral hepatitis was excluded by means of serological testing. Shortly after presentation, HCC was diagnosed by means of ultrasound and CT scan in addition to elevated tumour marker AFP (1000 ng/mL). The patient declined all therapeutic options and decided for best supportive care near her hometown. Thus, no follow up on the patient's condition or course of HCC was available.

HCC patient 3

A 46-year-old female was admitted to our department for histologically confirmed HCC and liver cirrhosis (Child-Pugh-Score C) due to AIH. Sixteen years prior to presentation, the patient had already been treated for "non-A/non-B" hepatitis. Thus, retrospectively, a longer course of AIH must be assumed. Throughout the course of disease, treatment had been extended to prednisolone and azathioprine. An accompanying chronic viral hepatitis was excluded by means of serological testing. A CT scan demonstrated two nodes of 8.8 cm × 7.7 cm and 2.3 cm × 1.8 cm, confirmed to be HCC. Due to tumour size and lack of sufficient size of the potentially remaining liver after surgical resection, surgery was declined and the patient was offered palliative chemotherapy. However, the patient declined chemotherapeutic treatment and was lost in follow up.

RESULTS

In order to investigate the incidence of HCC in patients with AIH, we retrospectively examined our large cohort of 278 patients with AIH. Twenty-nine patients (10%) had been under treatment for AIH for less than one year. Thirty-two patients in the study cohort (11%) had cirrhosis at presentation. Of the remaining 255 patients, fifty-seven (22%) developed cirrhosis despite treatment at our department. Together, the total number of patients with cirrhosis and thus at risk for developing HCC was 89 (32%).

For these patients, a total of 431 patient-cirrhosis-years were evaluated. Median follow up time in patients with cirrhosis was 4.8 years (58 mo). Twenty-seven of these patients had a follow up with cirrhosis of more than five years. Overall, these patients were observed for an average of 8.6 years. Twenty-nine patients (10%) were under treatment for less than one year. During this period, none of the 278 patients developed HCC. Three more patients with HCC on the basis of liver cirrhosis due to AIH were referred to our department for treatment of HCC. However, the HCC did not develop during treatment or follow up within our study group. None of those patients seen for HCC in cirrhosis due to AIH tested positive for HCV or HBV or showed any signs of other chronic liver disease. Thus, these patients were excluded from our cohort for calculation of the incidence of HCC per patient-cirrhosis-years.

The 189 patients not developing liver cirrhosis during follow up were observed for a total of 1183 patient years, an average of 6.3 years. None of these 189 patients lacking histological evidence of cirrhosis developed HCC during the time of observation.

DISCUSSION

HCC is among the most common cancers worldwide and its incidence is rising. However, the incidence of HCC in patients with liver cirrhosis varies due to the underlying chronic liver disease. The risk of developing HCC due to chronic hepatitis was estimated to be approximately 1%-8% per patient year in patients with Hepatitis C[4-6,20,21] and 1%-15% in patients with hepatitis B[8]. Incidence in patients with alcoholic liver disease or hemochromatosis was reported to be somewhat lower, but still at the lower margin of the risk of patients with chronic viral hepatitis[9-11]. Finally, HCC incidence due to late stage primary biliary cirrhosis (PBC) was also reported to be up to 2% per patient year in late stage (stage Ⅳ) PBC[12,13].

Contrary to the number of reports on patients with chronic viral hepatitis, only very few cases of patients with HCC developing due to AIH have been reported. Furthermore, most of these patient reports were published before the era of HCV screening and thus, many of these may be accounted for by a HCV co-pathogenesis[15]. At present, besides individual case reports, only a single larger patient cohort has been published, definitely excluding HCV co-pathogenesis[14]. Park *et al*[14] demonstrated a low incidence of HCC in patients with AIH and cirrhosis. They observed only one HCC in 88 patients with cirrhosis due to AIH and in a total of 212 patients, pointing towards an incidence of HCC in patients with liver cirrhosis of about 0.1%. Comparing these numbers to patients with cirrhosis due to chronic viral hepatitis, a significant difference in the occurrence of HCC in cirrhotic livers due to these different entities becomes obvious.

In our cohort of 278 patients with AIH, 89 (32%) patients suffered from liver cirrhosis. During the period of follow up, none of the patients of our own cohort developed HCC. Three more patients with HCC on the basis of cirrhosis due to AIH were referred to our department for treatment of HCC (tertiary referral center for treatment of HCC). However, these patients were referred to our department already with the definite diagnosis of HCC or suspected liver cancer and reason for referral was treatment for HCC. Thus, the existence

of these three patients further confirmed the potential occurrence of HCC in patients with AIH-associated liver cirrhosis. Nevertheless, the patients were not included for the calculation of the incidence of HCC per patient-cirrhosis-years. Therefore, incidence of HCC in patients with cirrhosis due to AIH must be estimated to be less than 0.2% per patient-cirrhosis-year, since HCC was not observed in our cohort in 431 patient years.

Most importantly, all three patients seen for HCC in cirrhosis due to AIH tested negative for HCV or HBV. In two patients no signs of chronic liver disease other than AIH were diagnosed. However, patient 1 was known to suffer from overlap to PBC. Thus, we confirmed that development of HCC may occur independently of co-infection with any form of chronic viral hepatitis or other common causes for liver cirrhosis.

Our results were in accordance with the results published by Park *et al*[14], which primarily demonstrated a significantly lower incidence of HCC in patients with AIH. Although our total patient number was larger than the Park study[14], we had comparable numbers of cirrhosis in our patients, i.e. 89. These patients were observed for a total of 431 years. Median follow up was 4.8 years, which was less than the Park study. Since to our knowledge, the study by Park *et al*[14] still remains the only data on a comparably large cohort, evidence for an altered pathomechanism for HCC development in cirrhosis due to AIH must be considered.

Although the pathogenesis behind this significant difference in HCC development remains to be further elucidated, it may hold important insight into the pathomechanism of HCC differentiation. The lower incidence of HCC in AIH patients is even more surprising since AIH patients are commonly treated with immunosupressants such as steroids and azathioprine, potentially increasing the risk of malignant transformation. However, immunosuppressants such as steroids act through suppressing cytokines, important for inflammation. An increased production of some these cytokines, IL-1β and TNF-α, have been demonstrated to coincide with the presence of liver cancer[22]. Thus, downregulation of these cytokines by immunosuppressive therapy[23,24] may be speculated to contribute to protection from the development of HCC.

In conclusion, we characterized our large collective of patients with AIH and were able to demonstrate and confirm a low incidence of HCC in these patients. The further characterization of patients with HCC due to autoimmune hepatitis may finally lead to the identification of new therapeutic targets preventing the development of liver cancer and thus novel therapeutic strategies.

COMMENTS

Background

At present only very few cases of hepatocellular carcinoma (HCC) in patients with autoimmune hepatitis (AIH) and definite exclusion of chronic viral hepatitis have been published, suggesting that HCC due to AIH is rare.

Research frontiers

Compared to other causes of liver cirrhosis such as chronic viral hepatitis, alcohol, or hemochromatosis, the incidence of HCC due to AIH is significantly lower. From our data it was estimated to be less than 0.2% per patient-cirrhosis-year.

Innovations and breakthroughs

This study confirms that AIH patients have a lower risk of HCC than patients with other chronic liver disease, despite their often long term treatment with immunosuppressants.

Applications

Immunosuppressants such as steroids act through suppressing cytokines, essential mediators in inflammation. An increased production of some of these cytokines, IL-1β and TNF-α, has been demonstrated to coincide with the presence of liver cancer. Thus, downregulation of these cytokines by immunosuppressive therapy may be speculated to contribute to protection from the development of HCC. Identifying the differences in patients with AIH and other chronic liver diseases may lead to the identification of protective mechanisms against liver cancer.

Peer review

Compared to other causes of liver cirrhosis such as chronic viral hepatitis, alcohol, or hemochromatosis, the incidence of HCC due to AIH is significantly lower. From our data it was estimated to be less than 0.2% per patient-cirrhosis-year. This study confirms that AIH patients have a lower risk of HCC than patients with other chronic liver disease, despite their often long term treatment with immunosuppressants.

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BRIEF ARTICLES

Gastroenterology service in a teaching hospital in rural New Zealand, 1991-2003

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Abstract

AIM: To retrospectively collect inpatient and outpatient data and to assess the use of endoscopic procedures during the years 1991, 1997 and 2003 to analyse for trends.

METHODS: This retrospective survey was conducted in a University-associated Gastroenterology Unit offering secondary and tertiary health care services for a population of approximately 182 000 people in Southern New Zealand. Data collected included patient contacts (in- and outpatients), gastroscopic and colonoscopic investigations.

RESULTS: We observed a significant increase in the absolute numbers of patient contacts over the years (1991: 2308 *vs* 1997: 2022 *vs* 2003: 2783, $P < 0.0001$) with inflammatory bowel disease, other diseases of the colon, anus and rectum and iron studies related disorders decreasing significantly but liver disease and constipation increasing linearly over time. The use of endoscopy services remained relatively stable but colonoscopic investigations for a positive family history of colorectal cancer increased significantly while more gastroscopies were performed for unexplained anaemia.

CONCLUSION: The whole spectrum of gastroenterology contacts was studied. A substantial proportion of colonoscopies and outpatient consultations were undertaken to screen for colorectal cancer. This proportion is likely to grow further. Our findings have implications for the recruitment and training of the next generation of gastroenterologists.

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Key words: Colonoscopy; Colorectal cancer; Disease trends; Endoscopy; Gastroenterology; Hepatitis; Inflammatory bowel disease; Recruitment; Workforce

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INTRODUCTION

The worldwide demand for gastroenterological services is growing. In order to adapt a training curriculum for future gastroenterologists and to allocate health resources, disease trends and the workload of gastroenterologists need to be monitored closely[1-3]. The literature on this topic is sparse. In 1973, Switz first described the practice of gastroenterologists in Virginia, USA[4] but only a few studies followed, mainly focussing on specific procedures or diagnoses. Manning *et al* (1980) surveyed the practice of a single gastroenterologist[5] and in 1984 an analysis of the working patterns of 500 members of the American Gastroenterological Association was published, focussing mainly on functional disorders[6]. A more general profile of a district hospital gastroenterology service in the United

Kingdom was obtained by Holmes *et al* in 1987; there was little change over a 12 year period[7]. The most recent survey of practice patterns was conducted in the USA by Russo *et al* and was published in 1999[8].

However, each of these surveys had limitations and was therefore not suitable for generalisation. The Southern Island of New Zealand is mostly rural with approximately 182000 people scattered over an area of about 32000 sq km and is therefore comparable to many other regions outside the large population centres of Asia, Europe and the Americas. A difficult and often unmet task is to ringfence health resources (funding, staffing and facilities) prospectively so that the health needs of the population can be met. Long distances and a borderline population size make the justification for investments difficult. Projected data for disease and demand trends are of utmost importance. There is no published New Zealand data on the workload of gastroenterologists and the use of endoscopy procedures.

The goals of this study were (1) to retrospectively collect inpatient and outpatient data and (2) to assess the use of endoscopic procedures during the years 1991, 1997 and 2003 to examine the demographics of patients seen by the service and to analyse trends in diseases seen during these periods.

MATERIALS AND METHODS

Study population

This survey was conducted in the Dunedin Public Hospital Gastroenterology Unit. The hospital serves as the only secondary and tertiary public referral centre for a population of approximately 182000 people mainly in the Otago Region of the South Island of New Zealand. The majority of people in this area identify themselves as European in ethnic origin, with 5.9% identifying themselves as Maori, 3.1% as Asian, and 1.5% as Pacific Islanders[9]. These numbers were relatively stable over the observed period. The next advanced public health care centres are to the north or to the south and approximately 4 h by car.

The Gastroenterology Unit is usually staffed by one full-time consultant, two part time (0.5) consultants and one full-time registrar. Patients have access through referrals from local general practitioners and hospital specialists. The unit offers in- and outpatient specialist consultations and diagnostic procedures such as upper and lower diagnostic and therapeutic endoscopies, endoscopic retrograde cholangio-pancreatico-graphy (ERCP), trans-abdominal ultrasound, liver biopsy, and pH telemetry. Patients in general have the possibility to access private services but transfer to a public centre out of area is not possible.

Prior to the commencement of this study, ethical approval was obtained from the Lower South Regional Ethics Committee. The data collection was performed in two phases by two separate groups of final year medical students as part of their health-care evaluation projects during their attachment to the Department of Preventive and Social Medicine. Each phase included data from the same years: 1991, 1997 and 2003.

Data collection

The absolute number of patient contacts (i.e. individual patients might have been seen more than once) including inpatients (IP; first and subsequent admissions) and outpatients (OP; as new or follow-up visits) was determined for the years concerned. Disease trends were determined based on the diagnosis on first presentation of the disease. Excluded from analysis were deceased patients as their files had been destroyed, and those with missing files. At the time of our analysis in 2005/2006, 23.9% of all patients first seen in 1991 were deceased, compared to 10.4% and 4.1% first seen in 1997 and 2003, respectively. The proportion of missing data ranged from 1% to 4% for the three years combined.

Data was obtained via the hospital computerised patient management system. In addition, OP diagnoses were obtained by retrieving the relevant clinic letters from the hospital's medical record filing system.

Diagnostic data were in the form of International Classification of Disease Version 9 (ICD-9) codes (1991 and 1997) and ICD-10 codes (2003), providing all the diagnoses recorded during each admission. The ICD-9 codes were matched electronically to the ICD-10 codes so as to simplify analysis. When more than one gastrointestinal diagnosis was present in the raw data, it was ranked numerically by clinical coders but not necessarily in order of relevance to the clinical presentation (primary *vs* secondary diagnosis). Therefore, two investigators went through all the diagnoses listed for each admission, and chose the most likely primary and secondary diagnoses for that presentation. This resulted in more diagnoses than patients or patient contacts. If numbers for an entity were too small for analysis, codes were combined into broader diagnosis groups.

Endoscopic data were collected for all patients using the gastroenterology database and the computerised patient management system. Data on ERCP (n = 309), abdominal ultrasound and pH telemetry were not included in this analysis due to the small numbers of these procedures.

Statistical analysis

Differences between years for qualitative variables were tested using a chi-squared test or, in the case of small expected counts, Fisher's exact test. If the chi-squared test was statistically significant, linear trends were investigated using the Cochrane-Armitage (C-A) test for trend. Quantitative data was compared using analysis of variance (ANOVA). The level of significance for all tests was set at 5% and all tests were 2-sided. All statistical analyses were performed in STATA (Version 9.2) or XLSTAT (Version 2008).

RESULTS

In- and outpatient demographic features

There was a statistically significant increase in the absolute numbers of patient contacts (IP-first admissions and subsequent admissions and OP-new patients and

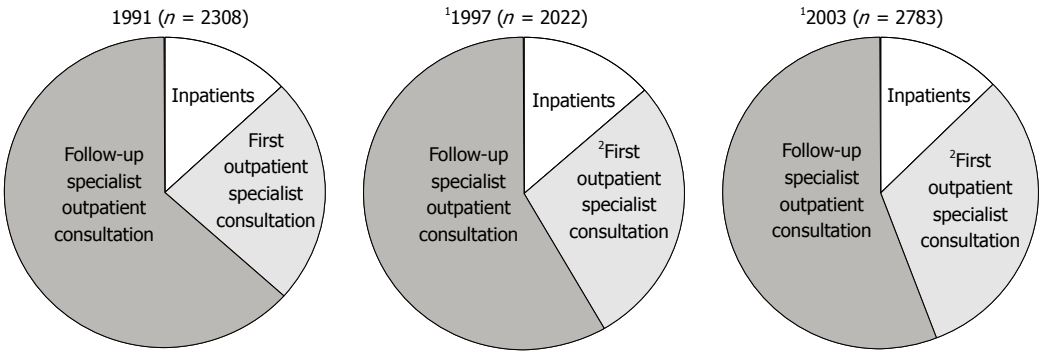

Figure 1 Total numbers of patients seen as in- and outpatients. There is a significant increase in the total number of patients ([1]C-A $P < 0.0001$) and in the number of new patients seen ([2]C-A $P < 0.0001$). The number of follow-up patients and inpatients remained statistically unchanged.

Table 1 Patient numbers and demographics

	Yr		
	1991	**1997**	**2003**
Inpatients			
No. of admissions[1]	173	310	391
Rate per 10 000 population[2]	9	16	20
Age mean (SD)[3]	54.1 (19.7)	55.9 (18.9)	53.4 (19.1)
Female *n* (%)	97 (56)	196 (63)	210 (54)
Outpatients			
No. of admissions[1]	553	469	756
Rate per 10 000 population[2]	30	25	39
No. of patients with information available	399	404	716
Age mean (SD)[3]	49.0 (19.3)	47.0 (18.0)	50.1 (18.5)
Female *n* (%)	246 (62)	256 (63)	421 (59)

[1]First hospital admissions and first specialist consultations showed a statistically significant linear increase over the years ($P < 0.001$ for both); [2]Estimates of the Otago population obtained from the Ministry of Social Development website: http://www.socialreport.msd.govt.nz/regional/r-councils/otago.html; [3]There was a statistically significant difference in the mean age of inpatients and outpatients ($P < 0.001$).

follow-up visits) over the years (Figure 1: 1991: 2308; 1997: 2022; 2003: 2783; C-A test $P < 0.0001$). Also, first hospital admissions and first specialist consultations (patients not seen before by this service) showed a statistically significant increase over the thirteen years between 1991 and 2003 (C-A test $P < 0.001$ for both, Table 1). For the three years combined, more females were seen as both in- and outpatients (58% inpatients and 61% outpatients were female, $P < 0.001$ for both). Overall, there was no statistically significant difference in the percentage of females seen in the in- and outpatient setting ($P = 0.123$). The mean age of inpatients was higher than that of outpatients ($P < 0.001$).

Diagnoses of in- and outpatients

In the IP (Table 2) setting, the top 5 diagnostic groups over all the years studied were: liver disease and abnormal liver function tests (LFTs; 20.8%), biliary disease (16.1%), inflammatory bowel disease (IBD; 11.7%), malignancy (8.7%) and other diseases of the colon/anus/rectum (5.0%). In comparison, the top 5 diagnoses in OP (Table 2) were: liver disease and abnormal LFTs (11.4%), other functional disease (11.1%), abdominal pain of unknown cause (10.0%), constipation (7.3%) and oesophagitis/gastro-oesophageal reflux disease (GORD; 6.5%). The detailed results are presented in Table 2. The data excludes patients referred by open access for endoscopy.

Disease trends

The number (percentage) of diagnoses seen in the inpatient setting is presented in Table 2. Disease trends in IP showed a statistically significant linear decline over time for inflammatory bowel disease, other diseases of the colon, anus and rectum and for iron studies related disorders as a proportion of all diagnoses (C-A tests $P = 0.015$, 0.040 and < 0.001, respectively). Conversely, liver disease and constipation increased linearly over time (C-A tests $P = 0.010$ for both). The proportion of patients seen with biliary disease spiked in 1997 but then decreased again in 2003 to levels similar to those seen in 1991, therefore, the difference across the years for biliary disease was not statistically significant (chi-square test $P = 0.502$). There was no statistically significant difference in the proportions of malignant disease (chi-squared test $P = 0.457$).

The number (percentage) of diagnoses seen in the OP setting is presented in Table 2. The OP diagnosis of abdominal pain of unknown cause and other functional disease showed a statistically significant linear decrease over the years (C-A tests $P < 0.001$ for both). Conversely, liver disease and abnormal LFTs, family history of bowel cancer, constipation and iron studies related disorders showed a statistically significant linear increase (C-A tests $P = 0.001$, < 0.001, 0.003 and 0.002, respectively) (Table 2). All other diagnoses in the in- and outpatient settings showed no statistically significant linear trend over the years studied.

Use of endoscopies

The number of procedures and the patient demographics are presented in Table 3. Over the three years studied, a total of 6705 procedures were performed; 4795 (71.5%) were gastroscopies and 1910 (28.5%) were colonoscopies.

There was no statistically significant linear trend across the years for either procedure (C-A tests $P = 0.379$ and 0.392, respectively). Overall, there were no gender differences for both colonoscopies and gastroscopies ($P = 0.132$ and 0.078, respectively). The age distribution

Table 2 Number (percentage) of diagnoses seen in the inpatient and outpatient setting[1]

Diagnosis	1991	1997	2003	Total
Inpatient				
Liver disease (including Hepatitis)and Abnormal LFTs[4]	33 (15.1)	90 (20.3)	124 (23.5)	247 (20.8)
Biliary disease	22 (10.1)	91 (20.5)	78 (14.8)	191 (16.1)
Inflammatory bowel disease[3]	38 (17.4)	47 (10.6)	54 (10.2)	139 (11.7)
Malignancy	23 (10.6)	34 (7.7)	47 (8.9)	104 (8.7)
Other diseases of colon/anus/rectum[3]	13 (6.0)	30 (6.8)	17 (3.2)	60 (5.0)
Abdominal pain unknown cause	12 (5.5)	9 (2.0)	35 (6.6)	56 (4.7)
Iron studies related disorders[3]	14 (6.4)	18 (4.1)	7 (1.3)	39 (3.3)
Constipation[4]	4 (1.8)	5 (1.1)	29 (5.5)	38 (3.2)
Pancreatic disease	7 (3.2)	11 (2.5)	17 (3.2)	35 (2.9)
Infective	5 (2.3)	14 (3.2)	8 (1.5)	27 (2.3)
Oesophagitis/GORD	4 (1.8)	9 (2.0)	8 (1.5)	21 (1.8)
Peptic ulcer disease	0 (0)	4 (0.9)	10 (1.9)	14 (1.2)
Irritable bowel syndrome	3 (1.4)	2 (0.5)	1 (0.2)	6 (0.5)
Haemorrhoids	0 (0)	1 (0.2)	1 (0.2)	2 (0.2)
Other functional disease	0 (0)	1 (0.2)	0 (0)	1 (0.1)
Other gastroenterological diagnosis[2]	31 (14.2)	67 (15.1)	82 (15.6)	180 (15.1)
Non-Gastroenterological diagnosis	9 (4.1)	11 (2.5)	9 (1.7)	29 (2.4)
Total	218 (100)	444 (100)	527 (100)	1189 (100)
Outpatient				
Liver disease (including Hepatitis) and Abnormal LFTs[4]	29 (6.4)	61 (13.5)	108 (12.9)	198 (11.4)
Other functional disease[3]	70 (15.5)	60 (13.3)	63 (7.6)	193 (11.1)
Abdominal pain unknown cause[3]	82 (18.2)	36 (8.0)	55 (6.6)	173 (10.0)
Constipation[4]	23 (5.1)	26 (5.8)	78 (9.4)	127 (7.3)
Oesophagitis/GORD	27 (6.0)	22 (4.9)	64 (7.7)	113 (6.5)
Irritable bowel syndrome	26 (5.8)	24 (5.3)	60 (7.2)	110 (6.3)
Family history of bowel cancer[4]	13 (2.9)	25 (5.5)	70 (8.4)	108 (6.2)
Other diseases of colon/rectum/anus	23 (5.1)	39 (8.6)	46 (5.5)	108 (6.2)
Inflammatory bowel disease	19 (4.2)	23 (5.1)	47 (5.6)	89 (5.1)
Haemorrhoids	16 (3.5)	15 (3.3)	47 (5.6)	78 (4.5)
Iron studies related disorders[4]	10 (2.2)	4 (0.9)	41 (4.9)	55 (3.2)
Infective (excluding Hepatitis)	8 (1.8)	26 (5.8)	10 (1.2)	44 (2.5)
Biliary disease	8 (1.8)	12 (2.7)	14 (1.7)	34 (2.0)
Malignancy	4 (0.9)	3 (0.7)	12 (1.4)	19 (1.1)
Peptic ulcer disease	9 (2.0)	1 (0.2)	8 (1.0)	18 (1.0)
Pancreatic disease	2 (0.4)	4 (0.9)	7 (0.8)	13 (0.8)
Other gastroenterological diagnosis[2]	73 (16.2)	52 (11.5)	78 (9.4)	203 (11.7)
Non-Gastroenterological diagnosis	9 (2.0)	18 (4.0)	26 (3.1)	53 (3.0)
Total	451 (100)	451 (100)	834 (100)	1736 (100)

[1]Number of diagnoses adds to more than the number of inpatients as a patient could have more than one diagnosis. The top 5 diagnostic categories per year and overall are highlighted; [2]This is a grouping of all the other minor diagnostic categories seen; [3]A statistically significant linear decrease over the years [Inpatient: Inflammatory bowel disease, other diseases of colon/anus/rectum and iron studies related disorders (P = 0.015, 0.040 and < 0.001, respectively); Outpatient: Abdominal pain of unknown cause and other functional disease (P < 0.001 for both)]; [4]A statistically significant linear increase over the years [Inpatient: Liver disease and constipation (P = 0.010 and 0.001, respectively); Outpatient: Liver disease, family history of bowel cancer, constipation and iron studies related disorders (P = 0.001, < 0.001, 0.003 and 0.002, respectively)].

Table 3 Procedure numbers and patient demographics

	Yr		
	1991	1997	2003
Colonoscopy			
No. of procedures	638	580	692
Rate per 10000 population[1]	34	31	36
Age mean (SD)	57.3 (14.5)	58.2 (15.0)	59.4 (14.7)
Female *n* (%)[2]	319/632 (50)	295/537 (55)	349/692 (50)
Gastroscopy			
No. of procedures	1439	1812	1544
Rate per 10000 population[1]	77	96	80
Age mean (SD)	58.9 (17.9)	58.1 (17.5)	58.4 (18.6)
Female *n* (%)[2]	726/1432 (51)	824/1595 (52)	793/1540 (51)

[1]Estimates of the Otago population obtained from the Ministry of Social Development website: http://www.socialreport.msd.govt.nz/regional/r-councils/otago.html; [2]Denominator given as data on sex was not available for all patients.

for colonoscopies changed over time (P = 0.027) while the age distribution for gastroscopies did not change (P = 0.527).

Indications and findings

The patient indications and findings following the colonoscopies are illustrated in Figures 2 and 3, respectively. The indications and findings following the gastroscopies are illustrated in Figures 4 and 5, respectively. Some patients had more than one finding so the percentages added up to more than 100% for each of the years.

Indications for colonoscopy

Most colonoscopies were performed to investigate rectal bleeding or as part of the surveillance for colorectal cancer and polyps in patients with a positive family history. Interestingly, while there was a further statistically significant increase in colonoscopies performed for cancer surveillance and for patients with a family history of cancer (C-A tests P = 0.014 and P < 0.001, respectively), there was a statistically significant decrease in the frequency of colonoscopies performed for rectal bleeding (C-A test P < 0.001), change in bowel habit (C-A test P < 0.001), and IBD surveillance/ulcerative colitis (C-A test P = 0.018) over the years studied. There was a tendency towards an increase in colonoscopies for unexplained anaemia (chi-squared test P = 0.057). The remaining indications showed no statistically significant linear trend (Figure 2).

Colonoscopy findings

Most colonoscopies were either reported as normal or showed polyps or diverticulae. Over the study timeframe, statistically significant increases in cancer (C-A test P = 0.007) and diverticulae (C-A test P < 0.001) were seen. In contrast, IBD decreased (C-A test P < 0.001), and there was a tendency towards an increase in polyps (C-A test P = 0.057). The proportion of normal findings did not change across the years (chi-squared test P = 0.493) (Figure 3).

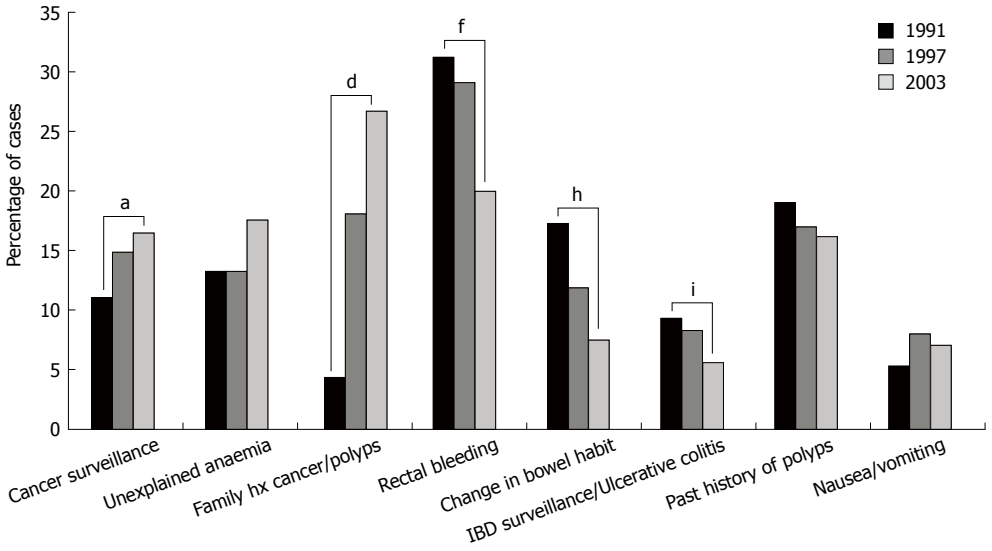

Figure 2 Indications for colonoscopies over the three years studied. There was a significant increase in colonoscopies in patients with a past medical history of colorectal cancer ($^{a}P = 0.015$) and in patients with a positive family history of colorectal cancer and/or polyps ($^{d}P < 0.001$). But there was a significant decrease in colonoscopies for rectal bleeding ($^{f}P < 0.001$), change in bowel habit ($^{h}P < 0.001$), and IBD surveillance ($^{i}P < 0.019$).

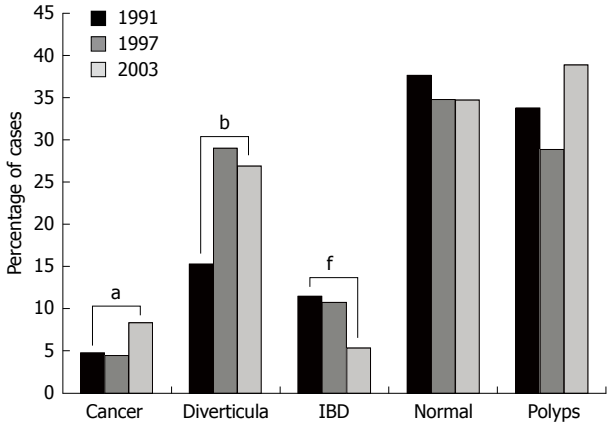

Figure 3 Colonoscopic findings. Over the 13 years studied, significant increases in cancer ($^{a}P = 0.008$) and diverticulae ($^{b}P < 0.001$) were seen. In contrast, findings of inflammatory bowel disease decreased ($^{f}P < 0.001$).

Indications for gastroscopy

Over the study timeframe, significantly more gastroscopies were performed for unexplained anaemia and heartburn (C-A test $P < 0.001$ for both), GI bleeding (C-A test $P = 0.034$), dysphagia (C-A test $P = 0.014$) and cancer surveillance (C-A test $P = 0.010$). Conversely, the frequency of gastroscopies carried out for nausea/vomiting, abdominal pain and healing surveillance decreased (C-A test $P < 0.001$ for all). There were no significant changes in the proportion of gastroscopies performed for dyspepsia (chi-squared test $P = 0.114$) (Figure 4).

Gastroscopy findings

Most gastroscopies showed only inflammatory changes. The number of ulcers, healed ulcers and scarring/strictures (C-A test $P < 0.001$) for all) and the proportion of normal findings (C-A test $P = 0.005$) decreased significantly over the years. There was no significant difference in Barrett's oesophagus (chi-squared test $P = 0.313$) and cancer (chi-squared test $P = 0.424$) observed over time. Findings of inflammatory changes and hiatus hernia differed significantly across the years (chi-squared $P < 0.001$ and 0.005, respectively), but there was no significant linear trend across the years (C-A test $P = 0.148$ and 0.478, respectively) (Figure 5).

DISCUSSION

Our retrospective analysis over 13 years (1991-2003) aimed to collect data on in- and outpatient demographics, disease trends and endoscopic procedures in a secondary and tertiary but rural referral centre in southern New Zealand. This analysis has produced data which can be used for prospective planning in order to allocate health resources and in curriculum development for the future gastroenterological workforce.

Our study has several interesting findings, demonstrating that the working pattern for gastroenterologists over the last 13 years has changed dramatically in several aspects. This was not only true for absolute numbers of patient contacts, but also for indications and findings. These results may permit speculation on possible trends. However, this analysis was not aimed at explaining observed disease trends in the wider context as this is very complex and needs to take both contractual arrangements as well as local characteristics into account.

The absolute numbers of patient contacts by this service increased significantly over time, despite a stable workforce both inside and outside the hospital (e.g. consultants and general practitioners). This was mainly seen for in- and outpatient contacts, while the number of investigations remained stable over the years studied. Our results show distinct differences in the types of conditions seen in inpatient versus outpatient settings (Table 2). The gastroenterology service continues to see consistently more females (60%) than males (inpatients and outpatients; $P < 0.001$), in contrast to the 50:50 ratio seen in the study by Bohra in Ireland[10]. Age distributions have also remained stable over the thirteen year observation period, with a mean age of 48.9 years for outpatients and 54.4 years for inpatients ($P < 0.001$).

Excluding endoscopies, the gastroenterology service sees approximately 660 patients for a first specialist

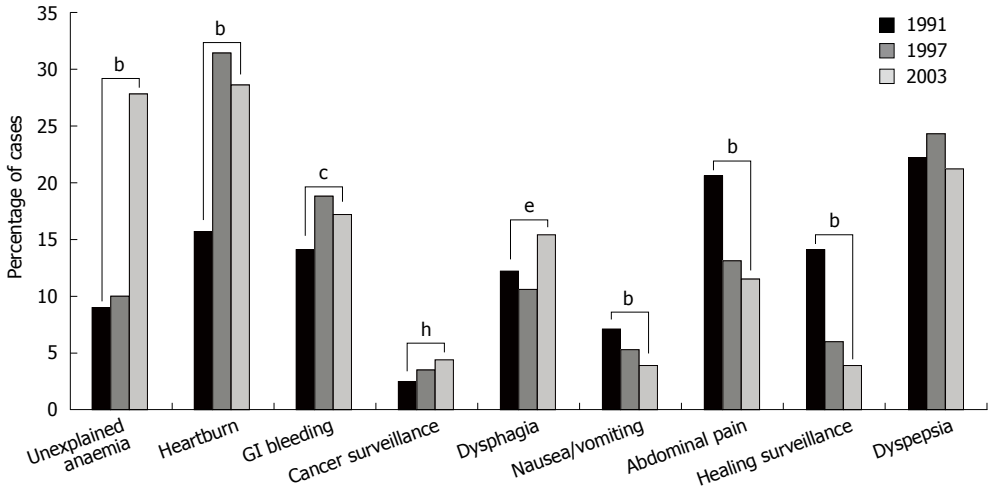

Figure 4 Indications for gastroscopies over the three years studied. There was a significant increase in gastroscopies performed for unexplained anaemia and heartburn ($^bP < 0.001$), as well as for suspected upper gastrointestinal bleeding ($^cP < 0.034$), dysphagia ($^eP = 0.014$) and cancer surveillance ($^hP = 0.01$).

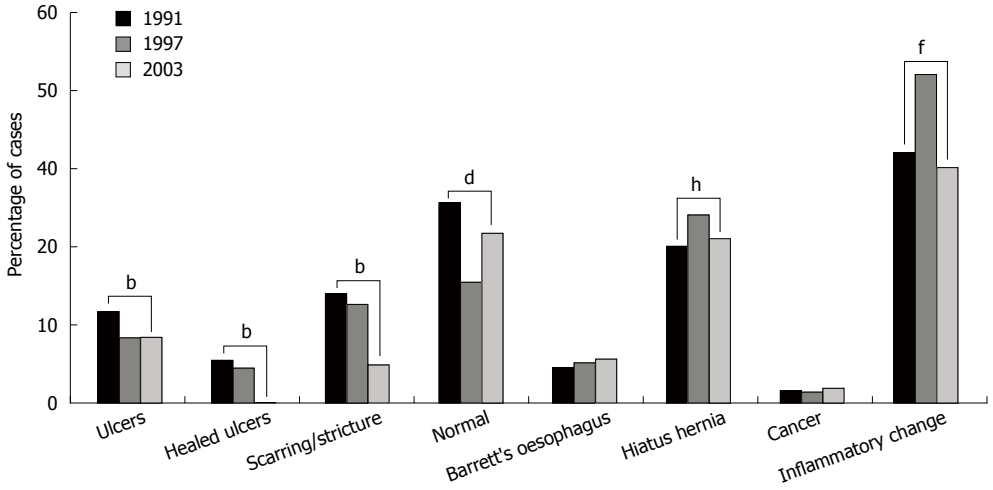

Figure 5 Gastroscopic findings. The number of ulcers, healed ulcers and scarring/strictures ($^bP < 0.001$) for all) and the proportion of normal findings ($^dP = 0.006$) decreased significantly over the years. Findings of inflammatory changes and hiatus hernia fluctuated significantly across the years (chi-squared $^fP < 0.001$ and $^hP < 0.005$, respectively) ($^fP = 0.148$ and $^hP = 0.478$, respectively).

assessment each year. The top five OP diagnoses (liver diseases, functional disorders, abdominal pain, constipation and reflux) were similar across the three years studied. This is largely in agreement with statistics published by Russo *et al* ranking abdominal pain, reflux, gastroenteritis, gastritis, haemorrhoidal disease and irritable bowel disease first but with liver disease in 16th place[11]. A survey among 376 members of the American Gastroenterological Association revealed Irritable Bowel Syndrome (19%) as the most common diagnosis, followed by oesophageal reflux and peptic disorders (17% and 10%, respectively), IBD (14%) and liver diseases (11%)[8]. If we summarise abdominal pain and constipation as a likely presentation of a functional disorder, this indication made up 27.3% and is therefore very much in agreement with the above survey by Russo *et al*[8]. However, in contrast to the view by Powell[1], but in agreement with others[5-7], there were significantly fewer patients with functional disorders compared with the beginning of the period. It is difficult to speculate on a reason for this finding, however, due to long waiting times of up to 6 mo for non-urgent referrals it is likely that only treatment-resistant cases are seen by this service.

Patients with liver disorders made up 8.5% of our outpatients and over 20% of the inpatient population with a significant increase seen over time. This might be a reflection of treatment options for Hepatitis C (testing available since 1992 in NZ), and the understanding of the significance of non-alcoholic fatty liver disease (NAFDL). However, most of these patients are admitted as day cases for liver biopsies and are therefore not truly reflecting inpatients. Such a trend was suspected by Powell[1] and in part confirmed by Russo *et al*[8].

We found that the absolute number of endoscopies (gastroscopies and colonoscopies) performed in our centre remained largely the same over the study period, approaching 80/10 000 gastroscopies and 36/10 000 colonoscopies in 2003 with 77/10 000 and 34/10 000 in 1991, respectively. This was largely due to contractual constraints. Scott and Atkinson in their 5-year review found that the annual number of gastroscopies doubled in the early 80's and was expected to reach 120/10 000 in the 90's with a similar trend expected for colonoscopies[2,12]. The Working Party of the Clinical Services Committee of the British Society of Gastroenterology stated in 1991 that an annual requirement of 100/10 000 gastroscopies and 20/10 000 colonoscopies were needed for a district general hospital[13].

In a study by Westbrook, gastroscopies were recorded at 179/10 000 population in 1997/98 in New South Wales, Australia[14]. Our almost stable lower numbers for endoscopic services can have several explanations and

might not necessarily reflect the actual demand. Due to a strictly limited budget, the service in Dunedin can offer open access for gastroscopy but not for colonoscopy. Colonoscopy referrals undergo rigorous evaluation before an investigation is granted. Throughout the years the percentage of normal colonoscopies remained stable at approximately 35%. While it is relatively easy to judge the appropriateness of an investigation retrospectively, prospectively this task is difficult. It has been reported that the indication for up to 24.5% of colonoscopies[15] and 15-39% of gastroscopies[16-18] might not be appropriate. In contrast, we observed a significant increase in the number of cases of colorectal cancer at colonoscopy. This has to be seen on the background of more than a doubling of the incidence of colorectal cancer between 1956 and 1996 from 32/100 000 to 80/100 000 in male New Zealanders[19]. A significant relative increase in colonoscopies performed for cancer surveillance and for a positive family history of colorectal cancer (from 20 in 1991 to 168 in 2003) and also for unexplained anaemia was also observed reflecting our increasing understanding of preventative measures in medicine.

The relative increase in gastroscopies performed for unclear anaemia is probably a result of our greater understanding of the changing face of coeliac disease[20,21]. The awareness of the significance of Barrett's oesophagus in the development of oesophageal cancer[22] is possibly reflected in the increased number of endoscopies performed for dysphagia and cancer surveillance. However, we did not find an increased incidence of Barrett's oesophagus (approximately 5.5%-6.8%)[16] or cancer of the upper gastrointestinal tract (1.5%-1.2%) over the years.

In summary, we have presented data that reflect changes in the working pattern of a rural tertiary gastroenterology service over 13 years. Most of our findings are in agreement with those of previous studies. However, this analysis is unique in that we studied the whole spectrum of gastroenterology contacts in a hospital providing secondary and tertiary services. This study provides dynamic information to aid allocation of health resources and emphasises the importance of preventive medicine and also workforce development. A substantial proportion of colonoscopies and outpatient consultations are already undertaken to screen for colorectal cancer in an at-risk population. This proportion is likely to grow further, and New Zealand is no exception. In the United States, about 13% of the population is over 65 years, and projections estimate this figure may rise to 20% over the next twenty years[23]. Our study showed that the median age of patients undergoing endoscopy in Dunedin Public Hospital was almost 60 years, and our aging population means that more patients will require endoscopic procedures in the coming years.

A larger workforce will probably be necessary to meet these increasing demands. A recent review of the gastroenterology workforce available in the United States suggests that there will be a significant shortfall in gastroenterologists by 2010[24]. The baby boom generation is not only providing more patients, but those gastroenterologists who are also part of that era are now nearing retirement. In 1997, about 30% of American gastroenterologists were within 5-10 years of retirement[23]. All of this has huge implications for the recruitment and training of the next generation of gastroenterologists.

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COMMENTS

Background

The field of gastroenterology in line with other areas in medicine is rapidly changing. New insight into disease progression and pathomechanisms translate into daily medicine. In order to obtain a high standard of patient care, gastroenterological training as well as recruitment has to adapt to these changes.

Innovations and breakthroughs

This analysis is unique in that the whole spectrum of gastroenterology contacts in a hospital setting providing secondary and tertiary services was studied. The Dunedin Public Hospital is located in the Otago region which is in general, sparsely populated. Nevertheless, advanced gastroenterological services are expected to meet the demand of the people. Most data is provided by large specialised centres, not suitable for units based in rural settings. However, it is these centres that need to consider trends to carefully plan training and staffing.

Applications

This study provides dynamic information to aid allocation of health resources and emphasises the importance of preventive medicine and also workforce development. Our findings have implications for the recruitment and training of the next generation of gastroenterologists and a larger workforce will be necessary to meet these increasing demands.

Peer review

This clinical study gives a well structured survey about gastroenterological diagnoses in the University-associated Hospital in Southern New Zealand. The manuscript is well written and all data are statistically verified.

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BRIEF ARTICLES

Biochemical markers for non-invasive assessment of disease stage in patients with primary biliary cirrhosis

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Abstract

AIM: To evaluate different biochemical markers and their ratios in the assessment of primary biliary cirrhosis (PBC) stages.

METHODS: This study included 112 patients with PBC who underwent a complete clinical investigation. We analyzed the correlation (Spearman's test) between ten biochemical markers and their ratios with different stages of PBC. The discriminative values were compared using areas under receiver operating characteristic (ROC) curves.

RESULTS: The mean age of patients included in the study was 53.88 ± 10.59 years, including 104 females and 8 males. We found a statistically significant correlation between PBC stage and Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) to platelet ratio (APRI), ALT/platelet count, AST/ALT, ALT/AST and ALT/Cholesterol ratios, with the values of Spearman's rho of 0.338, 0.476, 0.404, 0.356, 0.351 and 0.325, respectively. The best sensitivity and specificity was shown for AST/ALT, with an area under ROC of 0.660.

CONCLUSION: Biochemical markers and their ratios do correlate with different sensitivity to and specificity of PBC disease stage. The use of biochemical markers and their ratios in clinical evaluation of PBC patients may reduce, but not eliminate, the need for liver biopsy.

INTRODUCTION

Primary biliary cirrhosis (PBC) is a slowly progressive autoimmune disease of the liver that primarily affects women. Its peak incidence occurs in the fifth decade of life, and it is uncommon in persons under 25 years of age. PBC is diagnosed more frequently than it was a decade ago because of its greater recognition by physicians, the widespread use of automated blood testing and the antimitochondrial antibody test, which is relatively specific for the diseases[1,2]. Histologically, PBC is characterized by portal inflammation and immune-mediated destruction of the intrahepatic bile ducts. These changes occur at different rates and with varying degrees of severity in different patients. The loss of bile ducts leads to decreased bile secretion and the retention of toxic substances within the liver. This results in further hepatic damage, fibrosis, cirrhosis, and eventually, liver failure[3].

Liver biopsy is necessary to assess the extent of liver damage in an anicteric patient, e.g. in a patient in whom a transplantation is considered because of heavy symptom load. A liver biopsy may also be desirable to detect cirrhosis indicating a need for esophagogastroscopy for the detection of oesophageal varices or it may reveal a relative contraindication for treatment with

ursodeoxycholic acid, which is effective only in early stages of PBC[4,5].

For both, the physician and the patient, the decision to proceed with a liver biopsy is not trivial one. Furthermore, many patients are reluctant to experience repeated biopsies, which limits our ability to monitor disease progression and the effects of treatments. Significant complications, defined as requiring hospital admission or prolonged hospital stay, occurs in 1%-5% of patients who have a liver biopsy, while the reported mortality rate varies between 1:1000 and 1:10 000[6,7]. Most, but not all, studies have shown that the incidence of complications is related to both the presence of relative contraindications and the number of biopsies taken[6,8]. Despite these reservations, needle liver biopsy has been used as the "gold standard" for the assessment of liver fibrosis. However, a review of the available data on the accuracy of needle liver biopsy to define the stage of fibrosis reveals that the assessment of liver fibrosis is affected by significant sampling and interpretative errors. Therefore, while liver biopsy remains the "gold standard", both the clinician and the researcher should view the result of liver biopsy with some reservation and should interpret the findings in the broader clinical context. These problems must be considered when assessing the performance of other non-invasive tests for fibrosis when needle biopsy is used as the "gold standard". Many fibrosis experts would therefore consider serum fibrosis tests with an ROC area of 0.85-0.90 to be as good as liver biopsy for staging fibrosis.

The aim of our study is to evaluate different biochemical markers and their ratios in assessment of various stages of PBC[9].

MATERIALS AND METHODS

This study includes 112 patients investigated and treated for primary biliary cirrhosis in Clinic for Gastroenterology and Hepatology, Clinical Centre of Serbia during 2006. The diagnosis was based on clinical features, laboratory test, imaging diagnostics, and whenever possible, on liver histology.

The following information was collected for each patient: age, gender, biochemical parameters [aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, ALT to platelet ratio (APRI), AST/ALT ratio, ALT/AST ratio, ALT/alkaline phosphatase (AF) ratio, ALT/gamma glutamyl transferase (GGT) ratio, ALT/cholesterol (Chol) ratio], and the histological stage of the disease.

Stage I -Ⅳ classification of the disease was applied by an experienced pathologist who assessed the liver biopsies. Stage I is defined by the localization of inflammation to the portal triads. In stage Ⅱ, the number of normal bile ducts is reduced, and the inflammation extends beyond the portal triads into the surrounding parenchyma. Fibrous septa link adjacent to portal triads in stage Ⅲ, while stage Ⅳ represents the end-stage liver disease, characterized by obvious cirrhosis with regenerative nodules[10].

Laboratory testing and needle biopsy of livers were performed within a maximum of one week.

The Ethics Committee of our institution approved the study and all patients gave informed consent prior to inclusion in this investigation.

All collected data were analyzed and correlated. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS®, version 14.0). Basic descriptive statistics included means, standard deviations, ranges and percentages. For correlation analysis, we used Spearman's test. Differences were considered statistically significant if the two-tailed *P* value was less than 0.05. Confidence intervals and the best cut-off values for the assessment of disease stage were calculated using ROC curves.

RESULTS

The study included 112 patients with PBC who underwent a complete clinical investigation. The mean age of patients included in the study was 53.88±10.59 years. (range 29-76), 104 females and 8 males. There were 58 (51.8%) patients in PBC stage Ⅰ, 20 (17.9%) in stage Ⅱ 20, 24 in stage Ⅲ (21.4%), and the remaining 10 patients (8.9%) were in stage Ⅳ, characterized by liver cirrhosis. In fact, 78 patients (69.7%) had a mild form of PBC (stages I and Ⅱ), while 34 patients (30.3%) showed an advanced form of the disease (stages Ⅲ and Ⅳ). Values of analyzed biochemical markers, as well as results of statistical analyses are presented in Table 1. We found a statistical significant correlation between PBC stage and AST, APRI, ALT/platelet, AST/ALT, ALT/AST and ALT/Chol ratio, with the values of Spearman's rho of 0.338, 0.476, 0.404, 0.356, -0.351 and 0.325, respectively. The best sensitivity and specificity was shown for AST/ALT, with the area under ROC of 0.660.

DISCUSSION

During the last decade many studies have been designed to identify non-invasive markers capable of providing accurate information about liver fibrogenesis activity and stage of liver fibrosis in patients with chronic, potentially progressive hepatic diseases. The ideal characteristics of such markers are: (1) Specific for liver fibrosis; (2) Providing measurement of: (a) stage of fibrosis, (b) fibrogenesis activity; (3) Not influenced by comorbidities (e.g. renal, reticulo-endothelial); (4) Known half-life; (5) Known excretion route; (6) Sensitive; and (7) Reproducible. Two quite different approaches have been followed. Many studies have evaluated "direct" markers of fibrogenesis, i.e. of biochemical parameters, measurable in the peripheral blood as a direct expression of either the deposition or the removal of ECM in the liver. These direct markers of liver fibrosis include several glycoproteins (hyaluronan, laminin, human cartilage glycoprotein 39 (YKL-40), the collagens family (procollagen Ⅲ, type Ⅳ collagen and type Ⅳ collagen 7s domain), the collagenases and

Table 1 Values of biochemical markers and results of statistical analyses for differentiation of disease stage in patients with PBC

	mean ± SD	Range	Sensitivity (%)	Specificity (%)	Confidential interval	Area under ROC	Spearman's rho	*P* value
Aspartate aminotransferase (AST) (U/L)	69.41 ± 84.7	14-466	42.5	62.5	27.8-63.3	0.484	0.303	< 0.05
Alanine aminotransferase (ALT) (U/L)	87.39 ± 105.89	13-594	40	50	30.7-60.6	0.455	0.232	NS
Albumin	37.67 ± 4.69	27-47	27.5	62.5	23.8-54.3	0.391	0.117	NS
ALT to platelet ratio (APRI)	0.39 ± 0.42	0.06-2.16	42.5	50	23.7-58.7	0.412	0.351	< 0.01
AST to platelet ratio	0.33 ± 0.38	0.06-1.70	32.5	50	19.2-53.1	0.362	0.407	< 0.01
AST/ALT ratio	0.81 ± 0.23	0.51-1.38	47.5	75	17.0-50.3	0.660	0.347	< 0.01
ALT/AST ratio	1.32 ± 0.34	0.73-1.97	32.5	62.5	49.2-82.9	0.337	-0.297	< 0.05
ALT/ alkaline phosphatase (AF) ratio	0.42 ± 0.44	0.10-2.44	35	42.8	34.0-70.8	0.594	0.094	NS
ALT/gama glutamyl transferase (GGT) ratio	0.68 ± 0.94	0.12-5.09	40	56.2	33.0-67.0	0.502	0.38	NS
ALT/cholesterol (Chol) ratio	13.65 ± 10.87	2.5-59.76	35	50	26.0-61.6	0.438	0.285	< 0.05

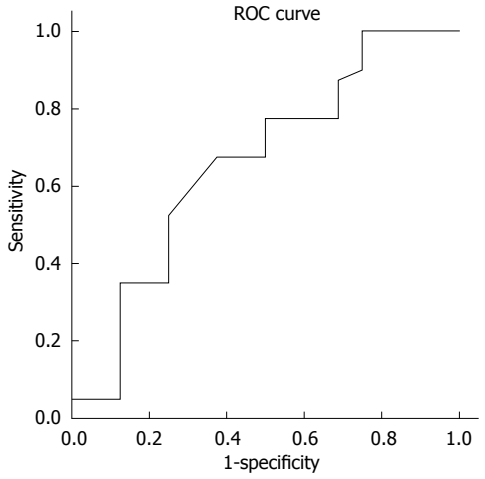

Figure 1 ROC for diagnosis of PBC disease stage using AST/ALT ratio.

their inhibitors (metalloproteinases and tissue inhibitors of metalloproteinases) and a number of cytokines connected with the fibrogenetic process (TGF-β1, TNF-β). A second and easier approach in the search of non-invasive markers of liver fibrosis has been choosing single or combined hematological and/or biochemical parameters that reflect the stage of liver disease, and assessing and comparing the accuracy of their diagnostic performance. This approach, using routinely performed blood tests, has led to the identification of sets of markers able to define the stage of liver fibrosis with accuracy very similar, if not superior, to that of the more sophisticated and difficult to test direct markers. The diagnostic performance of most direct and indirect markers of liver fibrosis has been investigated in all the common etiological forms of chronic liver diseases, including hepatitis C, hepatitis B and alcoholic and non alcoholic fatty liver disease and steatohepatitis, although some of them have been more extensively tested in patients with chronic hepatitis C[11].

There are many published articles on non-invasive assessment of fibrosis in patients with chronic hepatitis C infection, but only one published article[12] analyzed the value of AST/ALT ratio as an indicator of cirrhosis in patients with PBC. This study included 160 patients with PBC and for 121 they had laboratory data and liver histology. The authors of the study analyzed the clinical and laboratory data, as well as follow-up outcomes: liver-related death, liver transplantation and survival. The AST/ALT ratio was also used for assessment in alcohol-induced liver cirrhosis prediction of oesophageal varices and ascites presence. It is suggested that the AST/ALT ration increases in patients who develop liver cirrhosis, regardless of its cause. The reason for the increased AST/ALT ratio is unknown. It is suggested that the sinusoidal clearance of AST decreases in cirrhotic patients[13-15]. Nyblom *et al*[14] reported the use of this ratio for discrimination between cirrhotic and non-cirrhotic patients with sensitivity of 82% and specificity of 79%, for a cut-off value 1.1. The explanation for such high sensitivity is that a large number of cirrhotic patients were included, while sampling variability in the liver biopsies contributed to low specificity. The study concluded that the AST/ALT ratio is of clinical value as a predictor of cirrhosis in patients with PBC, but not as a prognostic factor.

Our study explored the value of different biochemical markers in the assessment of a range of disease stages in PBC patients. The sensitivity and specificity was calculated to discriminate early (stages I and Ⅱ) *vs* advanced (stages Ⅲ and IV) stages of PBC. We showed that several biochemical markers could be successfully used for staging the disease. Our results are, to some extent, comparable with the results of the previous similar studies, while the observed lower sensitivity (47.5%) and specificity (75%) for the AST/ALT ratio (Figure 1) could be explained by different study design.

New technology has been developed based on the fact that liver stiffness (LS) increases as liver fibrosis progresses[16]. The FibroScan 502 (FS, EchoSens, Paris, France) for transient elastography is a new modality developed for non-invasive evaluation of LS. LS correlated well with the histological stage of fibrosis. Changes in liver fibrosis stage may thus be estimated non-invasively using transient elastography[17,18]. Nevertheless, further studies are needed to confirm the value of this technique in different chronic diseases of liver.

In our opinion, the potential predictive value of aminotransferase and platelet count ratios in prediction of PBC stage may be used in evaluation of PBC evolution, despite their limited sensitivity and specificity, especially when considering their availability and cost effectiveness. Combination panels of non-invasive biomarkers may improve the accuracy of the single tests.

COMMENTS

Background

The main goal of this study was to evaluate different biochemical markers and their ratios in assessment of various stages of primary biliary cirrhosis (PBC).

Research frontiers

Despite liver biopsy remaining the "gold standard", there is great clinical interest in the use of non-invasive methods to assess disease stage in primary biliary cirrhosis.

Innovations and breakthroughs

This study explored the value of different biochemical markers in assessment of a range of disease stages in PBC patients. The sensitivity and specificity was calculated to discriminate early (stages Ⅰ and Ⅱ) *vs* advanced (stages Ⅲ and Ⅳ) stages of PBC. We showed that several biochemical markers could be successfully used for staging the disease.

Applications

Use of biochemical markers and their ratios in clinical evaluation of PBC patients may reduce, but not eliminate, the need for liver biopsy. The potential predictive value of aminotransferase and platelet count ratios in prediction of PBC stage may be used in evaluation of PBC evolution, despite their limited sensitivity and specificity, especially when considering their availability and cost effectiveness.

Peer review

This is a simple and elegant study that increases our knowledge of the non-invasive evaluation of biliary cirrhosis staging.

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BRIEF ARTICLES

Usefulness of bispectral monitoring of conscious sedation during endoscopic mucosal dissection

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Abstract

AIM: To assess the usefulness of bispectral index (BIS) monitoring in order to carry out endoscopic submucosal dissection (ESD) safely and with patients' satisfaction.

METHODS: Three hundred sixty-six patients with an early-stage neoplasm of the digestive tract were enrolled. The BIS monitor (A-1050: Aspect medical systems/NIHON KOHDEN, Tokyo, Japan) was used. The appropriate sedative condition was set at 55 to 75 BIS levels (BIS value) during the endoscopic procedures.

RESULTS: Among 366 cases, 13 were accompanied by adverse events during and/or after ESD. All episodes occurred in cases with BIS value between 56 and 65. Hypotension was observed in four cases, and bradycardia in six. Respiratory distress was observed in two cases with chronic pulmonary obstructive disease. All patients with adverse events were able to leave the hospital without extension of the hospitalization.

CONCLUSION: BIS monitoring is useful to safely perform ESD. A BIS value of 70 to 75 is suitable for ESD.

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Key words: Endoscopy; Cancer; Sedation; Propofol; CO_2

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INTRODUCTION

Endoscopic submucosal dissection (ESD) has recently been developed for *en bloc* resection of gastric cancer, which results in high tumor eradication rates, as well as a modality for the precise histological evaluation of the entire lesion[1,2]. Furthermore, recent studies have reported that ESD is useful for selected localized neoplasms of the esophagus and the colon[3,4]. However, ESD is a highly complex endoscopic technique and sometimes needs long time to be performed. So, conscious sedation is the standard of care for the majority of ESD cases to perform a safe procedure and to minimize complications.

The bispectral index (BIS) is a quantitative parameter of the hypnotic effects of anesthetic drugs on the central nervous system (CNS)[5,6]. BIS is not an instrument just for the frequent analysis of electroencephalogram, but it analyzes the relationship among the different components of the electroencephalogram in various phases. BIS also takes into account the correlation between the electrocardiogram and the CNS suppression. BIS is a dimensionless number ranging from 0 to 100, with 0 representing no cortical activity and 100 representing fully awake state. BIS has been utilized in guiding dosing of anesthetics, including decreasing the amounts of drug use and enhancing faster emergence and recovery[5]. To avoid excessive administration of propofol, we introduced the monitoring of the sedation depth by BIS, which enables the objective evaluation of the sedation.

It is desirable to make an objective judgment of the depth of the conscious sedation during ESD and to maintain a sedation that does not affect the cardiorespiratory dynamics. BIS monitoring is expected to avoid excessive administration of anesthetic drugs

during ESD. In this study, we investigated the usefulness of BIS monitoring while using propofol in the course of ESD. Propofol has been reported to afford a higher comfort and satisfaction to patients and to reduce the time to recovery, compared to previously reported drugs such as benzodiazepines and opiates[7,8].

MATERIALS AND METHODS

Patients

Three hundred sixty-six patients with early-stage esophagus cancer, gastric cancer and/or colorectal cancer, treated with ESD from May, 2004 to April, 2007 at the Hospital of the Shiga University of Medical Science (Otsu, Japan), were enrolled into the study. The baseline features and history of these patients are reported in Table 1.

Anesthesia

An anesthesist or a specialist in endoscopy trained in anesthesiology performed the procedure, including the administration of a sedative drug during operation. Two BIS sensors were put on the forehead of each patient and another sensor was placed on the outer side of an eyebrow. The BIS monitor (A-1050: Aspect medical systems/NIHON KODEN, Tokyo, Japan) was used. As soon as the sedative drug started its effects, oxygen (2 L/min) was given through a nasal cannula. Three-lead electrocardiogram, pulse oximetry and blood pressure were monitored.

Just before the sedation started, intravenous drip infusion of pentazocine (15 mg/body) was given. An initial 40-mg bolus of propofol was administered intravenously to all patients, followed by continuous infusion of propofol (3 mg/kg per hour). The appropriate sedative condition during endoscopic procedures was set at 55 to 75 BIS levels (BIS value). From May, 2004 to September, 2005, the BIS level was set between 56 and 65, while later on (i.e. from October, 2005 to April, 2007) it was set between 70 to 75. In order to maintain suitable BIS levels, the dosage of continuous infusion of propofol was adjusted.

Adverse events during and after ESD were recorded. We observed delayed awakening (i.e. the patient failed to awake 15 min after the stop of propofol administration), hypotension (systolic blood pressure < 90 mmHg), bradycardia (heart beats < 50/min) and respiratory failure (patient needing a mandatory ventilation).

RESULTS

Among the 366 cases, 13 were accompanied by adverse events during and after ESD (Table 2). The adverse events occurred during the period in which BIS value was set between 56 and 65 (12 cases out of 139 cases, 9%), but no adverse events occurred as long as the BIS value was set between 70 and 75.

Hypotension was observed in four cases. One of them occurred immediately after the administration of propofol, but in the other three it occurred at a later phase during ESD. These four patients recovered quickly when propofol was decreased or stopped, together with an adjustment of infusion of fluids.

Respiratory depression was observed in two cases, both of which had chronic obstructive pulmonary disease (COPD.) One patient had a history of chronic bronchial asthma, the other of pulmonary emphysema. During ESD, 2 to 5 L/min of oxygen was given and adjusted at a timely basis in order to maintain a $SpO_2 > 92\%$. The latter case developed CO_2 narcosis after ESD, but gradually recovered.

Bradycardia was observed in six patients. All of them recovered with an adjustment of fluid infusion.

All patients with an adverse event were able to leave the hospital without extending the duration of the hospitalization.

Table 1 Backgrounds of patients (number of cases)

BIS value	56-64 (May/2004 -Sep/2005)	70-75 (Oct/2005 -April/2007)	Total
Esophageal cancer	13	36	49
Gastric cancer	100	149	249
Colon cancer	26	42	68
Total	139	227	366

Table 2 Adverse events (number of cases)

BIS value	56-64 (May/2004 -Sep/2005)	70-75 (Oct/2005 -April/2007)	Total
Delayed awakening	1	0	1
Decreased blood pressure	4	0	4
Bradycardia	6	0	6
Respiratory depression	2	0	2
Total	13	0	13

DISCUSSION

The standard protocol of conscious sedation is generally determined by the experience of doctors at the respective medical institutions. Ideal conditions of conscious sedation are as follows: (1) rapid and short acting, (2) no accumulation and rapid recovery after operation, (3) no modulation of respiratory and cardiovascular dynamics, and (4) no hepatic or renal toxicity. In Japan, midazolam is widely used, while flumazenil is given as an antagonist after the operation[7]. However, because of the different half-life of these two drugs (midazolam, approximately 120 min; and flumazenil, approximately 50 min), there is a high risk of a new period of unconsciousness after the treatment.

Propofol is the most frequently used intravenous anesthetic in the field of intensive cares. The most important feature of propofol is its short half-life. Thus, even after prolonged administration, it does not bring about the repeated sedation once the administration is stopped. In a controlled study of propofol versus

midazolam, it has been reported that patients showed a higher level of satisfaction with propofol than with midazolam[7-9]. In the guidelines of endoscopy under sedation issued by the Society of American Gastrointestinal and Endoscopic Surgeons (SAGES), it is recommended to use propofol for endoscopic treatments of long duration, due to its dual advantage of a deep sedation and a quick awakening, compared to benzodiazepines and narcotic drugs. However, propofol may cause cardiorespiratory suppression. Since there are no appropriate antagonists available, it is necessary, in case of cardiorespiratory suppression induced by excessive propofol administration, to carry out cardiorespiratory supportive care with a ventilator until propofol is fully metabolized.

To avoid excessive administration of propofol, we introduced monitoring by BIS, which enables the objective evaluation of the sedation depth. BIS is not an instrument just for the frequent analysis of electro-encephalogram, but it also analyzes the relationship among the different components of the electroencephalogram at various stages. BIS is equal to 100 when the patient is fully awake and 0 when an isoelectric electroencephalogram is observed. It has been reported that BIS is useful to evaluate the sedation depth during digestive endoscopy or treatment[10].

During surgical operations under general anesthesia, the BIS value is usually set at 45 to 65[11]. Thus, we also set the target BIS during ESD at 56 to 64, at least during the initial period. However, since we experienced cases with adverse events during ESD, we slightly increased the BIS values. Thus, after October 2007, we set a BIS value at 70 to 75. Propofol has sedative effects but it does not possess analgesic properties. Therefore, we added pentazocine (15 to 30 mg) and in most cases we could carry out the ESD without impediments, using these BIS values.

There were four cases of decreased blood pressure during the first period. In one case, this occurred because of the bolus infusion of propofol at the time of induction of sedation. After this case, propofol was given through a syringe pump with constant speed. Also, we administrated propofol from the port which is located close to the peripheral intravenous indwelling needle, in order to minimize the lag time between the start of injection and the actual appearance of a sedative effect. By doing this, we did not experience any additional case of decreased blood pressure after the infusion.

Regarding the other three cases, decreased blood pressure occurred in the second half of ESD. Each of these patients had a history of elevated blood pressure and therefore they had been taking antihypertensive drugs until the day of ESD. Decreased blood pressure might be attributed to the interaction of propofol with antihypertensive drugs. However, all patients recovered quickly when propofol was decreased or stopped, or when the fluid infusion was adjusted. In addition, no additional case of decreased blood pressure was experienced in the later period, i.e. when the BIS value was set higher.

Two cases of respiratory depression were confirmed and one of the patients had a history of chronic bronchial asthma. Respiratory depression was due to accidental aspiration during ESD. This is not considered as an adverse event of propofol, and could be prevented by placing an aspirating cannula in the stomach. The other patient had a history of pulmonary emphysema and developed CO_2 narcosis because of the excessive infusion of oxygen during ESD. Therefore, based on this case, the oxygen infusion was started at 1 liter per minute when dealing with patients with COPD. Also, we made it a rule to carry out an arterial blood gas analysis at least once during ESD. As a consequence, we have not experienced any additional cases of respiratory depression.

Recent studies have reported that ESD is useful for localized esophagus and colon neoplasms[3,4]. ESD for such neoplasms is a much more complex endoscopic technique than that used for gastric neoplasms. So, BIS monitoring with conscious sedation may be useful for safely performing ESD of esophageal and colonic neoplasms.

In conclusion, BIS monitoring of conscious sedation with propofol is considered a very effective method for safe implementation of ESD. A BIS value between 70 to 75 is suitable for monitoring sedation depth during ESD.

COMMENTS

Background

Endoscopic submucosal dissection (ESD) is a highly complex technique for *en bloc* resection of localized neoplasms of the digestive tract, and conscious sedation is the standard of care to perform safe procedures. During ESD conscious sedation is required, but the usefulness of sedation monitoring has not been reported.

Research frontiers

The bispectral index (BIS) is a quantitative parameter of the hypnotic effects of anesthetic drugs on the central nervous system. In this study, we present data supporting the usefulness of BIS monitoring for safely performing ESD with patients' satisfaction.

Innovations and breakthroughs

This is the first study to report that conscious sedation by BIS monitoring is useful for ESD.

Applications

BIS monitoring may be useful during ESD of gastric, esophageal or colonic tumors.

Terminology

Endoscopic submucosal dissection (ESD) is a highly complex technique for *en bloc* resection of localized neoplasms of the digestive tract. BIS is a quantitative parameter of the hypnotic effects of anesthetic drugs on the central nervous system. BIS is not an instrument just for the frequent analysis of electroencephalogram, but it also analyzes the relationship among the different components of the electroencephalogram at various phases.

Peer review

This paper describes a novel method of assessing sedation during endoscopy and as such it should be published.

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BRIEF ARTICLES

Mutation of cytotoxin-associated gene A affects expressions of antioxidant proteins of *Helicobacter pylori*

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Abstract

AIM: To determine if disruption of the *cagA* gene of *Helicobacter pylori* (*H pylori*) has an effect on the expression of other proteins at proteome level.

METHODS: Construction of a *cagA* knock out mutant *Hp27*_Δ*cagA* (*cagA*$^-$) *via* homologous recombination with the wild-type strain *Hp27* (*cagA*$^+$) as a recipient was performed. The method of sonication-urea-CHAPS-DTT was employed to extract bacterial proteins from both strains. Soluble proteins were analyzed by two-dimensional electrophoresis (2-DE). Images of 2-DE gels were digitalized and analyzed. Only spots that had a statistical significance in differential expression were selected and analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS). Biological information was used to search protein database and identify the biological function of proteins.

RESULTS: The proteome expressions between wild-type strain and isogenic mutant with the *cagA* gene knocked-out were compared. Five protein spots with high abundance in bacteria proteins of wild-type strains, down-regulated or absently expressed in bacteria proteins of mutants, were identified and analyzed. From a quantitative point of view, the identified proteins are related to the *cagA* gene and important antioxidant proteins of *H pylori*, including alkyl hydroperoxide reductase (Ahp), superoxide dismutase (SOD) and modulator of drug activity (Mda66), respectively, suggesting that *cagA* is important to maintain the normal activity of antioxidative stress and ensure *H pylori* persistent colonization in the host.

CONCLUSION: *cagA* gene is relevant to the expressions of antioxidant proteins of *H pylori*, which may be a novel mechanism involved in *H pylori cagA* pathogenesis.

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Key words: *Helicobacter pylori*; *Cytotoxin-associated gene A*; Knock-out; Antioxidant protein; Two-dimensional electrophoresis

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Huang ZG, Duan GC, Fan QT, Zhang WD, Song CH, Huang XY, Zhang RG. Mutation of cytotoxin-associated gene A affects expressions of antioxidant proteins of *Helicobacter pylori*. *World J Gastroenterol* 2009; 15(5): 599-606 Available from: URL: http://www.wjgnet.com/1007-9327/15/599.asp DOI: http://dx.doi.org/10.3748/wjg.15.599

INTRODUCTION

Helicobacter pylori (*H pylori*), a spiral-shaped bacterium that colonizes the human gastric mucosa, is estimated to inhabit at least half of the world's human population[1]. Infection with *H pylori* is associated with development of peptic ulcer, gastric carcinoma and gastric mucosa-associated lymphoid tissue lymphomas[2-4]. However, little is known about the molecular mechanisms of pathogenesis induced by *H pylori* and the fundamental causes of diversity in infection outcomes. Cytotoxin-associated gene A protein (CagA) is a *H pylori* immuno-

dominant antigen with its gene residing in the cag pathogenicity island, which is a 40-kilobase insertion containing genes involved in virulence[5]. Recent studies indicate that CagA is delivered from *H pylori* into the cytoplasm of *H pylori*-attached gastric epithelial cells via the type-Ⅳ secretion system[6]. Upon membrane localization, translocated CagA interacts with a number of host proteins involving cell signaling in a tyrosine phosphorylation-dependent and -independent manner[7,8]. Epidemiological studies have shown that *cagA*$^+$ positive *H pylori* strains are associated with higher grades of gastric mucosal inflammation as well as severe atrophic gastritis and gastric carcinoma[9,10]. CagA is considered a marker of increased pathogenic potential and may play an important role in the pathogenesis induced by *H pylori*.

China is one of the nations with the highest *H pylori* infection incidence[11]. More than 90% isolated strains possess *cagA* gene[12]. However, the biological activity of *cagA* gene still remains unclear. In the present study, we analyzed two related *H pylori* strains, *Hp27* and *Hp27_*Δ*cagA*, through a proteomic approach. Wild type strain *Hp27* is a *cagA* gene possessor, while strain *Hp27_*Δ*cagA* is an isogenic *cagA* knock out mutant. Proteins with altered expressions can be separated by two-dimensional electrophoresis (2-DE) and conclusively identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis of the peptide digests. This study was to determine if disruption of the *cagA* gene has an effect on the expression of other proteins of *H pylori* at proteome level.

MATERIALS AND METHODS

Bacterial strains

Hp27 (*cagA*$^+$) isolated from a patient with chronic atrophy gastritis was grown on Brucella agar plates containing 10% sheep blood supplemented with 10 mg/L vancomycin, 2500 U/L polymyxin B, 2 mg/L amphotericin and 5 mg/L trimethoprim, in an anaerobic jar consisting of 50 mL/L O_2, 100 mL/L CO_2, and 850 mL/L N_2 at 37℃ for 3 d. Kanamycin (25 mg/L) was added for mutated strains selection.

Construction of Hp27_ΔcagA isogenic mutant

Isogenic *Hp27_*Δ*cagA* mutant was obtained from strain *Hp27* as follows. In brief, we produced a *Hp27_*Δ*cagA* isogenic mutant harboring a total *cagA* deletion as previously described[13]. *H pylori* were grown on blood agar plates under microaerophilic conditions for 3 d and genomic DNA was extracted with a genomic DNA purification kit (Takara). An upstream (U fragment) and a downstream region (D fragment) of the *cagA* gene were amplified over the genomic DNA as homologous arms using the *P1*/*P2* and *P3*/*P4* primer pairs, respectively. A kanamycin-resistant gene was amplified as a screening marker from *pEGFP-N2* vector (Clontech) using the *P5*/*P6* primer pair. The primer sequences are as follows (restriction sites are underlined): *P1-Xho* Ⅰ: 5'-GCG<u>CTCGAG</u>ACTTTCTTGTAGCTGTC-3'; *P2-Hin*dⅢ: 5'-GGC<u>AAGCTT</u>TGTTTCTCCTTTACT-3'; *P3-Pst* Ⅰ: 5'-GG<u>CTGCAG</u>AGGATTGAGGAATAC-3'; *P4-Xba* Ⅰ: 5'-CG<u>TCTAGA</u>TTTTAGCGATCAAACAAC-3'; *P5-Hin*dⅢ: 5'-GC<u>AAGCTT</u>ATGATTGAACAAGATGGATTG-3'; *P6-Pst* Ⅰ: 5'-GG<u>CTGCAG</u>TCAGAAGAACTCGTCAAGAAG-3'.

After digestion of PCR products with *Xho* Ⅰ/*Hin*dⅢ (U fragment) and *Pst* Ⅰ/*Xba* Ⅰ (D fragment), the kanamycin resistance gene digested with *Hin*dⅢ/*Pst* Ⅰ was introduced between the U and D fragments. The resulting chimera was cloned into the *Xho* Ⅰ/*Xba* Ⅰ-digested *pBluescript SK Ⅱ* (-) vector (Stratagene) to obtain the construct targeting vector: *pBSK*Δ*cagA*_kan. *Hp27* served as a recipient strain was electrotransformated with *pBSK*Δ*cagA*_kan. Bacteria were grown on blood agar plates for 48 h and then streaked on fresh selective plates supplemented with 25 μg/mL of kanamycin. A real *cagA* isogenic mutant (*Hp27_*Δ*cagA*) was selected from kanamycin-resistant colonies and the corresponding insertional DNA region was checked by PCR to confirm the exact recombination.

Protein extraction

A wild-type strain of *Hp27* and an isogenic mutant of *Hp27_*Δ*cagA* were harvested from Brucella agar plates and then washed 3 times with ice-cold PBS. Total protein was extracted with an appropriate volume (300 μL) of lysis buffer containing 8 mol/L urea, 65 mmol/L DTT, 2% CHAPS, 2 mmol/L PMSF, 0.5% IPG buffer, and protease inhibitor mixture. The extraction mixture was sonicated with parameters of 120 W, 5 min, pulse: 1S, 2S. The protein mixture was centrifuged at 12000 r/min for 40 min. After transferred to a clean tube, the supernatant was stored at -70℃ as aliquots. The protein concentration was determined by Bradford dye-binding assay with bovine serum albumin as the standard.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was carried out as follows. Precast IPG strips (pH 3-10 linear, 18 cm, Amersham Pharmacia Biotechnology Inc.) were used in the first dimension. A total amount of 1000 μg protein was diluted to a total volume of 350 μL with the buffer containing 8 mol/L urea, 20 g/L CHAPS, 5 g/L IPG buffer 3-10, 20 mol/L DTT and a trace of bromophenol blue. After loaded on IPG strips, IEF was carried out on IPGphor (Bio-Rad,USA) according to the following protocol: rehydration for 16 h at 50 V, 1 h at 200 V, 1 h at 500 V, 1 h at 1000 V , 5 h at 10000 V and 60000 V h at 10000 V (total 112.5 kVh). The current was limited to 50 μA per gel. After IEF separation, the strips were immediately equilibrated for 2 × 15 min with an equilibration solution containing 50 mmol/L Tris-HCl (pH 6.8), 6 mol/L urea, 300 g/L glycerol and 20 g/L SDS. Then, 20 mmol/L DTT was included in the first equilibration solution, and 20 g/L iodoacetamide was added in the second equilibration step to alkylate thiols. Electrophoresis in the second dimension was carried out

on 15% SDS-PAGE gels (18 cm × 20 cm × 0.1 cm). The strips were held in place with 5 g/L agarose dissolved in a SDS/Tris running buffer and electrophoresis was carried out at a constant power (2.5 W/gel for 40 min and 15 W/gel for 5 h) using a Protean Ⅱ xi cell gel SDS-PAGE system (Bio-Rad, USA).

Image processing and analysis

After electrophoresis, gels were stained with Coomassie brilliant blue, equilibrated in a solution containing 500 mL/L methanol, 50 mL/L acetic acid and 25 g/L Coomassie brilliant blue R-250 for at least 2 h, and rinsed in 300 mL/L ethanol containing 70 mL/L acetic acid. Digitalized images were obtained by ImageScanner GS-800 (Bio-Rad, USA) scanning of the gels, and analyzed qualitatively and quantitatively by the PDQuest gel image analysis software 7.1 (Bio-Rad, USA). To determine variation, three gels were prepared for each sample. The computer analysis allowed automatic detection and quantification of protein spots as well as matching. The normalized volume of protein spots was used to analyze the differential level of protein expression. Only those spots that had a statistical significance in differential expression were selected for further investigation.

Protein identification by MALDI-TOF-MS

Differential protein spots were cut out from the gel. After being washed with 300 μL milliQ water for 15 min, each protein spot was decolorized with the successive action of 50 μL of 15 mmol/L potassium ferricyanide and 50 mmol/L sodium thiosulphate for 5-10 min. Faded gel pieces were dried in a vacuum centrifuge tube for 5 min. Cysteine reduction and alkylation were performed and incubated with 10 mmol/L DTT, 100 mmol/L NH_4HCO_3 at 56℃ for 1 h in the dark. Gel pieces were dried again and incubated with 50 mmol/L fresh iodoacetamide in 100 mmol/L NH_4HCO_3 at room temperature for 30 min and rehydrated in digestion buffer containing 20 μL of 12.5 μg/mL modified trypsin and 20 mmol/L NH_4HCO_3 for 30 min in ice. Excess liquid was removed and the gel pieces were digested continuously at 30℃ overnight (> 16 h). The resulting peptide mixture was extracted from the digested solution by centrifugation and resuspended in 10 μL of 50% CH_3CN and 0.1% trifluoroacetic acid (TFA) for 10 min at 30℃ on a shaking platform. Peptide mass maps were generated by Applied Biosystems Voyager System 6192 MALDI-TOF-mass spectrometry (ABI, USA). Peptide masses were analyzed using the MS-Fit search program (http://prospector.ucsf.edu/ucsfhtml4.0u/msfit.htm). The searching parameters were set up as follows: acquisition mass ranges 900-3500 Da, the mass tolerance was ± 0.5 Da; the number of missed cleavage sites was allowed up to 1; the minimum number of matched peptide was four; species was set as bacteria; and the searching range was within the experimental pI value ± 0.5 pH unit and experimental Mr ± 20%.

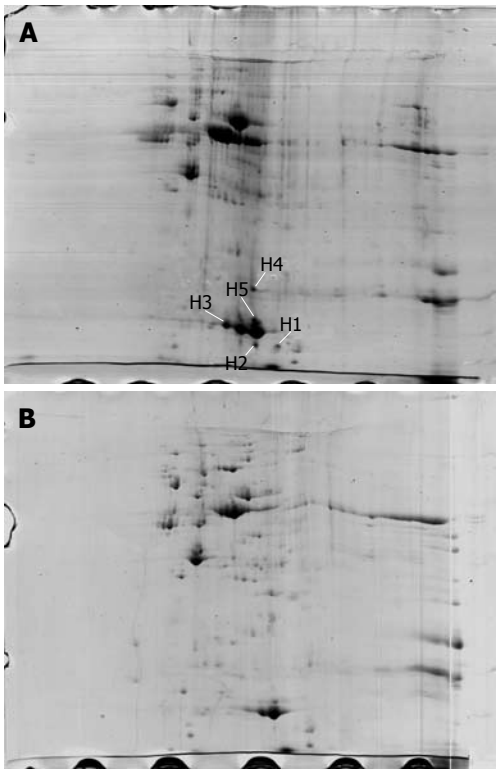

Figure 1 Differential two-dimensional maps of wild-type strain *Hp27* (A) and mutant strain *Hp27*_Δ*cagA* (B) stained with Commassie blue.

RESULTS

The proteome maps of *H pylori* strains *Hp27* and *Hp27*_Δ*cagA* were produced and compared (Figure 1). After spot detection, background subtraction, volume normalization, differentially expressed proteins were detected in wide-type strain versus isogenic mutant. The arrows indicate five protein spots whose expression levels were significantly differently represented in strain *Hp27* as compared to strain *Hp27*_Δ*cagA*.

Expression levels of the two proteins were obtained by calculating the relative spot volume of each protein *versus* the total amount of protein in the gel. Segments of 2-DE gel map for five proteins derived from strains *Hp27* and *Hp27*_Δ*cagA* are shown in Figure 2.

Five protein spots differentially expressed in strains *Hp27* and *Hp27*_Δ*cagA* were excised from 2-DE gels and identified by peptide mass fingerprinting (Figures 3 and 4).

Mascot searches using the peptide mass fingerprinting data indicated that the differentially expressed proteins were alkyl hydroperoxide reductase (Ahp), superoxide dismutase (Sod) and modulator of drug activity (Mda66) (Table 1). These three proteins are all important antioxidant proteins of *H pylori*. From a quantitative point of view, these proteins are novel, and have not been reported previously in relation to *cagA* gene.

DISCUSSION

A comparative proteome analysis was carried out between the two *H pylori* strains: *Hp27*, a wild-type

Table 1 Mascot search results of PMFs in 5 protein spots

Spot	Accession No.	MW	PI	Description	MOWS E score	Sequence coverage (%)
H1	gi|2313748	21591	6.59	Modulator of drug activity (Mda66)	62	46
H2	gi|2314747	22221	5.88	Alkyl hydroperoxide reductase (AhpC)	111	67
H3	gi|2314747	22221	5.88	Alkyl hydroperoxide reductase (AhpC)	140	76
H4	gi|2313490	24602	5.77	Superoxide dismutase (SOD)	86	62
H5	gi|2313490	24602	5.77	Superoxide dismutase (SOD)	132	69

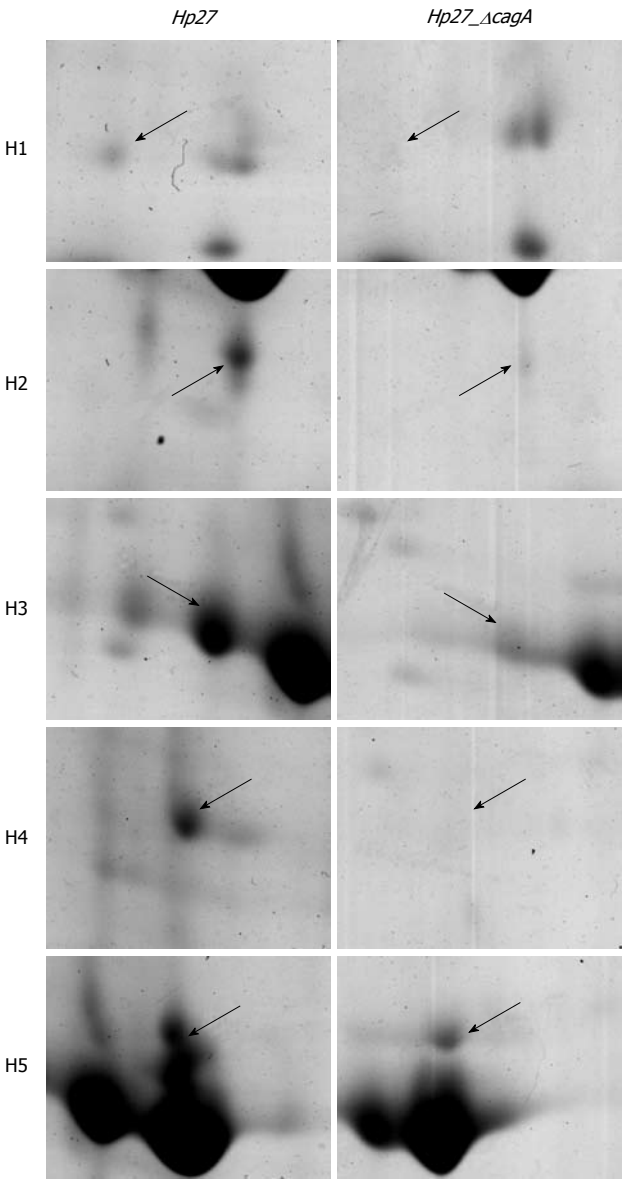

Figure 2 Magnified segments of 2-DE gel map of protein spots including H1~H5.

strain and *Hp27_Δcag*A, a *cagA*$^-$ isogenic mutant. Five differential protein spots, which are abundant in bacteria proteins of wild-type strains and are down-regulated or absently-expressed in bacteria proteins of mutants, were selected to perform in-gel trypsin digestion and MALDI-TOF-MS-based PMF analysis. The three identified proteins are Ahp, Sod and Mda66. Of the 5 position identifications, spots H2 and H3 were identified as the same protein Ahp, and spots H4 and H5 were also identified as the same protein Sod, indicating that these gene products are present as isoforms with post-translational modification[14].

Alkyl hydroperoxide reductase (AhpC), a thioredoxin (Trx)-dependent AhpC, is a member of the 2-Cys peroxiredoxins family (2-Cys Prxs). A group of thiol-specific antioxidant enzymes, which catalyze the reduction of hydrogen peroxide and organic hydroperoxides, are ubiquitous proteins that protect organisms from damage by reactive oxygen species[15]. *H pylori* are oxygen-sensitive microaerophilic bacteria, and contain many antioxidant proteins, among which AhpC is most abundant. The function of AhpC is to protect *H pylori* from a hyperoxidative environment by reducing toxic organic hydroperoxides[16].Wang *et al*[17] reported that mutant cells defective in AhpC are more sensitive to oxidative stress conditions, accumulate more free (toxic) iron, and suffer more DNA fragmentation compared to wild type cells. Olczak *et al*[18] tested the ability of strains with mutation in *ahpC* (encoding alkyl hydroperoxide reductase) to colonize the stomachs of mice, and showed that the mutant is clearly more sensitive than the parent strain to both oxygen and cumene hydroperoxide and unable to colonize mouse stomachs, whereas 78% of the mice inoculated with the parent strain become *H pylori* positive. Recently, Chuang *et al*[19] revealed that AhpC of *H pylori* acts as a peroxide reductase in reducing organic hydroperoxides and as a molecular chaperone in preventing protein misfolding under oxidative stress. Besides, AhpC could also influence the activity of other proteins. Catalase in *ahpC* mutant partially inactivated (approximately 50%) in comparison with the parent strain, indicating that organic hydroperoxides (the substrate of AhpC), which accumulate in *ahpC* mutant cells, are responsible for the inactivation of catalase[20]. In this study, the expression of AhpC was down-regulated in the *cagA* gene knocked-out mutant (Hp27_Δ*cagA*), suggesting that AhpC, one of the most important anti-oxidative stress proteins of *H pylori*, is related with the *cagA* gene.

Superoxide dismutase (SOD), a nearly ubiquitous enzyme in organisms exposed to toxic environments, is able to catalyze the conversion of superoxide radicals to hydrogen peroxide and molecular oxygen. Single SOD in *H pylori*, encoded by the *sodB* gene, has been suspected to be a virulence factor for this pathogenic microaerophile[21]. Seyler *et al*[22], who first isolated mutants with interruptions in the *sodB* gene, found that the *sodB* mutants are devoid of SOD activity, and more sensitive to O_2 and H_2O_2 for both growth and viability.

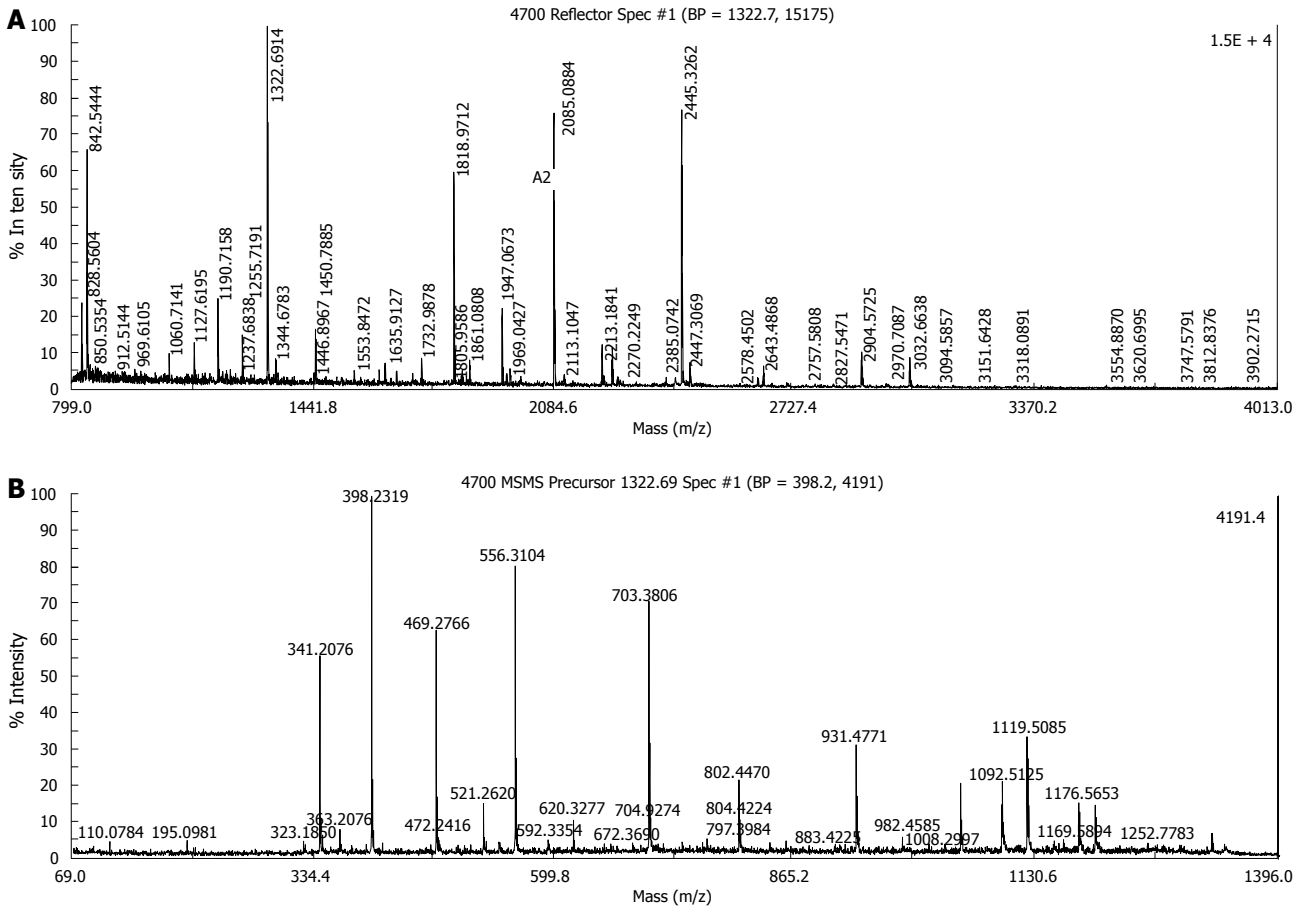

Figure 3 Mass spectrometry (MS) maps of one protein spots. A: MS of H1; B: MS/MS of H1.

Since oxidative stress is correlated with DNA damage, they studied the frequency of spontaneous mutation to rifampin resistance. The frequency of mutagenesis of the *sodB* mutant strain is about 15-fold greater than that of the wild-type strain. Wang *et al*[17] also reported that mutant cells defective in SOD are more sensitive to oxidative stress conditions and suffer more DNA fragmentation compared to wild type cells, and that a significant proportion of cells of *sodB* mutant strains develop into stress-induced coccoid form or lysed as well as that they also contain a significantly higher amount of 8-oxo-guanine associated with their DNA, compared to wild type cells. Seyler *et al*[22] observed that only 1 out of 23 mice inoculated with a SOD-deficient mutant of a mouse-adapted strain became *H pylori* positive, while 15 out of 17 mice inoculated with the wild-type strain harbored the organism, in a mouse colonization model, indicating that SOD is a virulence factor affecting the ability of *H pylori* to colonize the mouse stomach and is important for the growth and survival of *H pylori* under oxidative stress conditions.

Mda66, identified in the *ahpC napA* double mutant by two-dimensional gel electrophoresis combined with N-terminal protein sequencing, is another possible antioxidant protein[23]. Single bacterial homologue is a MdaB protein of *Escherichia coli* (*E. coli*), first identified as a modulator of drug activity (named *mda66*) because the gene is mapped at 66 min on the *E. coli* chromosome[24]. Wang *et al*[25] demonstrated that, like its homologue in *E. coli*[26], Mda66 protein is a NADPH quinone reductase and able to reduce quinone to quinol. Quinone metabolism within cells has a direct effect on the cell's ability to deal with oxidative stress[27]. Therefore, the reduced status of *H pylori* Mda66 protein plays an important role in the management of oxidative stress. Wang *et al*[25] reported that the wild-type strain could tolerate 10% oxygen, but the growth of *mdaB* mutant was significantly inhibited by 10% oxygen. The *mda66* mutant was also more sensitive to H_2O_2, organic hydroperoxides, and paraquat, an agent generated by superoxide. Although the wild-type strain survived more than 10 h after air exposure, exposure of the mutant strain to air for 8 h resulted in no recovery of viable cells. It was reported that oxidative stress sensitivity of *mda66* mutant can reduced the ability of *mda66* mutant to colonize mouse stomachs[25]. *H pylori* were recovered from 10 of 11 mouse stomachs inoculated with the wild-type strain, with about 5000-45 000 CFU/g of stomach, while only 3 of 12 mice inoculated with the *mdaB* mutant strain did not harbor any *H pylori*, and contained less than 2000 CFU/g of stomach[25]. Therefore, the physiological function of *H pylori* Mda66 protein is similar to that of NADPH quinone reductase that plays an important role in the management of oxidative stress and contributes to successful colonization of the host.

Oxidative stress resistance is one of the key properties enabling pathogenic bacteria to escape the effects of reactive oxygen produced in the host.

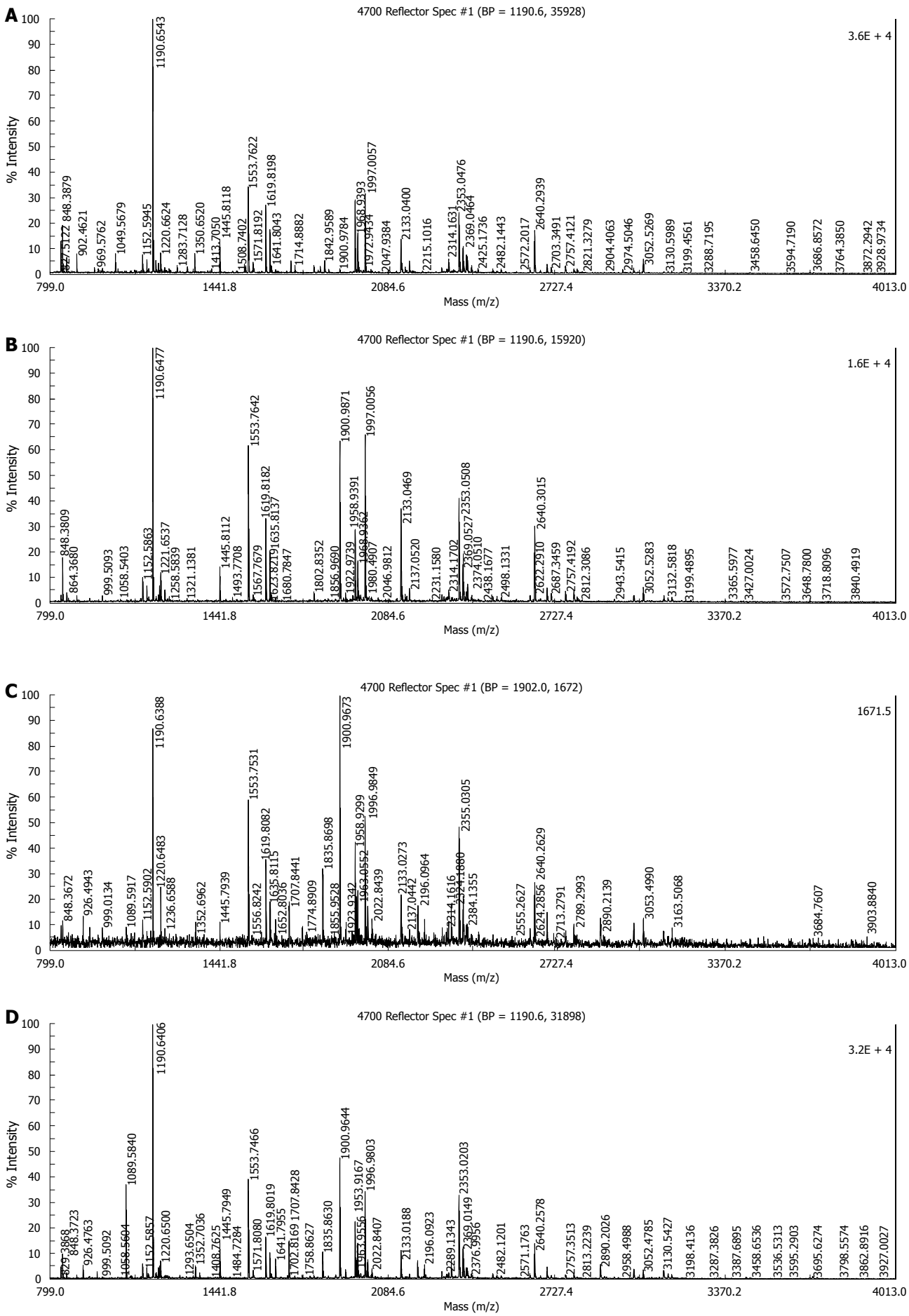

Figure 4 Mass spectrometry (MS) maps of 4 protein spots. A: MS of H2; B: MS of H3; C: MS of H4; D: MS of H5.

Therefore, proteins (enzymes) involved in oxidative stress resistance are the important factors for bacterial colonization and pathogenesis[28]. Microaero philic organisms, such as *H pylori*, are particularly vulnerable to the detrimental effects of oxygen and oxidative stress. Nevertheless, enzymes including AhpC, catalase (KatA), SOD, thioperoxidase (Tpx), *etc*., can maintain persistent infection by using a variety of protective enzymatic systems that eliminate or minimize toxic oxygen-derived products.

In this study, three important antioxidant proteins of *H pylori* including AhpC, SOD and Mda66 were identified from the 2-DE maps showing differentially expressed proteins between the wild-type strain (*Hp27*) and isogenic *cagA* knock out mutant (*Hp27_ΔcagA*), indicating that the *cagA* gene is relevant to the expressions of antioxidant proteins of *H pylori*. Disruption of the target gene was found to have a certain effect on the expression of other genes that its encoded protein was shown to play a direct or indirect role in the regulation of protein biosynthesis, suggesting that *cagA* gene quantitatively influences *ahpC*, *sodB* and *mda66* transcription or their subsequent translation and correct folding.

Regulatory genes are usually located in the genome upstream of genes. With reference to the genomic sequence of *H pylor 26695*, *cagA* is only located in the upstream of *mda66*, and it is difficult to explain how CagA regulates other antioxidant proteins. Since there are no DNA-binding motifs or motifs suggestive of a two-component regulatory system in CagA, the protein may act as a signal transducer by means of other proteins[8,29]. It was recently reported that a ferric uptake regulator (Fur) and a post-transcriptional regulator CsrA play a key role in the regulation of antioxidative stress enzymes[21]. We presumed that *cagA* might be related to Fur and/or CsrA, through which *cagA* influences the expression of antioxidant protein. However, the correlation between *cagA* and Fur and/or CsrA needs to be further studied.

In this study, *cagA* was found to be correlated with the three stress-resistant enzymes, suggesting that *cagA* gene may be of importance for *H pylori* to maintain the normal activity of antioxidative stress and to keep long-term persistence in the host, which is a novel mechanism involved in *cagA* pathogenesis. Based on our results and the reported linkage between *cagA* and motility[30], our conclusion is that *cagA* has virulence effects and may play a specific role in *H pylori* pathogenesis by influencing the expression of other proteins (enzymes).

COMMENTS

Background

Helicobacter pylori (*H pylori*) is a causative agent of gastritis, peptic ulcer and gastric cancer. CagA, a major virulence factor of *H pylori*, is considered a marker of increased pathogenic potential and may play an important role in the pathogenesis of *cagA* gene. However, little is known about the molecular mechanism and multiple biological functions of *cagA*.

Research frontiers

CagA is a *H pylori* immunodominant antigen whose gene resides in the *cag* pathogenicity island, a 40-kilobase insertion containing genes involved in virulence. A previous study showed that *cagA* is related with the motility of *H pylori*. However, its exact mechanism still remains unclear. In this study, a proteomic approach was employed to determine if disruption of the *cagA* gene has some effects on the expression of other proteins of *H pylori*, through which various functions of the *cagA* gene can be recognized.

Innovations and breakthroughs

A proteomic approach combined with MALDI-TOF-MS was employed to investigate changes in expression of the wild-type strain of *Hp27* (*cagA*$^+$) and the *cagA* gene knock out isogenic mutant. Three antioxidant proteins were identified, including alkyl hydroperoxide reductase (Ahp), superoxide dismutase (SOD) and modulator of drug activity (Mda66), respectively. It is implied that *cagA* is essential to maintain the normal activity of antioxidative stress and ensure *H pylori* persistent colonization in the host, which may be a novel mechanism involved in *cagA* pathogenesis.

Applications

These results provide a novel mechanism involved in *H pylori cagA* pathogenesis. The *cagA* gene may be of importance for *H pylori* to maintain the normal activity of antioxidative stress and keep long-term persistence in the host.

Terminology

CagA: Cytotoxin-associated gene A protein encoded by cytotoxin-associated gene pathogenicity island, a major virulence factor of *H pylori*. Two-dimensional electrophoresis: A two-step method to separate soluble proteins. Soluble proteins were first separated by isoelectric focusing (IEF) electrophoresis, and then according to the difference in molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Antioxidant protein: Proteins (enzymes) involved in oxidative stress resistance, which are important factors for bacterial colonization and pathogenesis.

Peer review

The results of this study are interesting. The findings are novel. However, how the absence of antioxidant proteins should be further studied.

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Role of color Doppler flow imaging in applicable anatomy of spleen vessels

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Author contributions: Xu WL was responsible for the research design, data extraction and statictics; Wang Y was responsible for the examination of ultrasound; Li M was responsible for the literature search; Li SL and Niu AG were responsible for the academic instructions.
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Abstract

AIM: To explore the role of color Doppler flow imaging (CDFI) in visualization of spleen vessels and to define its value for spleen micro-invasive operation.

METHODS: A total of 36 patients requiring laparoscopic splenectomy (LS) for various hematopathies and autoimmune diseases were randomly selected from April 2005 to May 2008. Anatomic types of spleen pedicle, adjacent relations between spleen vessels and pancreas, diameters of spleen artery and vein were detected and recorded by preoperative CDFI. Different operative procedures were performed according to different anatomic frameworks. The parameters were recorded by telerecording during LS and compared with those by preoperative CDFI using Chi-square test.

RESULTS: Two anatomic types of spleen pedicle and four different adjacent relations between spleen vessels and pancreas were detected by CDFI. The diameters of spleen artery and vein detected by CDFI were 0.46 ± 0.09 cm and 0.85 ± 0.35 cm, respectively. There was no statistical difference between the parameters recorded by CDFI and by telerecording (χ^2 = 0.250, 0.677, P > 0.05). LS was successfully performed following the anatomic information provided by preoperative CDFI.

CONCLUSION: Different anatomic frameworks of spleen vessels can be provided by preoperative CDFI, which instructs micro-invasive operation of spleen and increase the safety of operation.

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Key words: Ultrasound; Color Doppler imaging; Spleen; Anatomy; Laparoscopic operation

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INTRODUCTION

Laparoscopic splenectomy (LS) can relieve refractory hematopathies and autoimmune diseases[1]. However, LS is performed safely and effectively only by surgeons who are familiar with the anatomy of spleen vessels because it is vital to manage spleen pedicle successfully during LS[2-6].

With the advances in ultrasound techniques, color Doppler flow imaging (CDFI) has become one of the major diagnostic methods for vascular diseases especially for localization of target vessels. Furthermore, CDFI plays an important role in selecting the operational procedures for hematopathies[7]. In this study, the applicable anatomy of spleen vessels was examined in detail by CDFI before operation on patients requiring splenectomy in order to explore the value of CDFI in micro-invasive operation of spleen.

MATERIALS AND METHODS

Patients

Thirty-six consecutive patients (21 males and 15 females) with a mean age of 9.21 ± 4.06 years (range, 1.5-25 years) were randomly selected from April 2005 to May 2008. The indications for splenectomy were idiopathic thrombocytopenic purpura (n = 8), hereditary

spherocytosis (n = 19), portal hypertension and hypersplenia (n = 5), hemolytic anemia and hypersplenia (n = 3), and splenic neutropenia (n = 1). The study was approved by the institutional review board. All patients provided their informed consent.

Examinations of CDFI

All patients were carefully examined by an experienced ultrasonic physician with HDI-5000 color Doppler ultrasonograph (Philips Corporation). C_{5-2} abdominal probes were used with a 3.5 MH_Z mean frequency. Ultrasonic images were independently evaluated by one chief physician with 15-year experience in abdominal vascular imaging.

Patients were placed in a supine or lateral decubitus position and kept their stomach empty for 6-8 h before examination. Anatomic type of the spleen pedicle end, adjacent three-fourths of the distal segments of spleen vessels and pancreas, diameters of spleen artery and vein were measured, classified and recorded.

Surgical procedures

Accessory spleens were resected according to the preoperative ultrasonic examination. After splenculus ligament was separated by ultrasound shear, retinula cavity was opened. The surgeon separated and ligated the trunk of spleen artery directly if CDFI showed that all or parts of the segments of spleen artery were located at the anterosuperior border of pancreas or else, the surgeon separated the anadesma tissue around the pancreatic tail, then revealed and ligated the spleen artery if CDFI showed that most spleen artery segments were located behind the pancreas. The spleen pedicle was revealed and ligated. If the spleen pedicle was distributed, the surgeon cut off the secondary vessels. However, the trunk of spleen pedicle was cut off directly if it was magistral. Finally, all ligaments around the spleen were cut off and the whole spleen was resected. There were no intra-operative complications. Anatomic parameters of spleen were recorded.

Comparisons

Imaging findings were used to assess the preoperative strategy and retrospectively compared with operative findings.

Statistical analysis

The number and constituent ratio of anatomic parameters recorded by preoperative CDFI were compared with those by intra-operative telerecording. $P < 0.05$ was considered statistically significant.

RESULTS

Imaging results of CDFI

By adopting cross section below left costal margin, CDFI detected four different adjacent relations between three-fourths of the distal segments of spleen vessels and pancreas and two anatomic types of spleen pedicle end. The number and constituent ratio of the different anatomic parameters are listed in Table 1. The appearance of each anatomic parameter on CDFI was described and classified as types 1-4 (Figure 1A-D) and distributed and magistral types (Figure 2A-B). In type Ⅰ, the limpid spleen arterial blood stream signal was detected constantly in front of the distal pancreatic body or tail and eventually the signal increased and distributed into spleen like a tree (Figure 1A). In type Ⅱ, the color blood stream signal put forth from the rear of the pancreatic body passed by the pancreatic tail and went up or down to the hilum of spleen (Figure 1B). In type Ⅲ, the blood stream signal was detected in front of the pancreatic body and suddenly disappeared in the rear of pancreatic tail (Figure 1C). In type Ⅳ, the obvious blood stream signal was not detected in front of pancreas although the probe was completely removed. Nevertheless, the vague color signal was found leading to spleen behind pancreas (Figure 1D). In distributed type, two or three color signals of scattering blood stream were detected leading to the hilum of spleen beside the pancreatic tail without signal of main blood stream (Figure 2A). In magistral type, the limpid color signal of main blood stream led to the hilum of spleen beside the pancreatic tail but the evident signal of branches was hard to be detected (Figure 2B).

Table 1 Comparison of parameters recorded by preoperative CDFI and intra-operative telerecording n (%)

Anatomic parameters	CDFI	Intra-operative telerecording	χ^2	P
Anatomic types of the end of spleen vessels				
Distributed type	23 (63.9)	25 (69.4)	0.25	0.617
Magistral type	13 (36.1)	11 (30.6)		
Adjacent relationships between the distal three-fourths segments of spleen vessels and pancreas				
Type Ⅰ	16 (44.5)	17 (47.2)	0.677	0.879
Type Ⅱ	8 (22.2)	6 (16.7)		
Type Ⅲ	5 (13.9)	4 (11.1)		
Type Ⅳ	7 (19.4)	9 (25.0)		

The diameters of spleen artery and vein detected by CDFI were 0.46 ± 0.09 cm (range, 0.40-0.60 cm) and 0.85 ± 0.35 cm (range, 0.50-1.60 cm), respectively.

Comparison of parameters recorded by preoperative CDFI and intra-operative telerecording

To compare the number and constituent ratio of the parameters recorded by CDFI and telerecording during LS; there was no significant statistical difference between them (Table 1).

DISCUSSION

CDFI is the latest diagnostic technique for the detection of heart and blood vessels without trauma by using Doppler principle combined with B model imaging and M model echocardiogram. Furthermore, CDFI can display the direction and relative velocity of blood stream and provide temporal and spatial information of blood vessels[7]. With a higher sensitivity and accuracy of

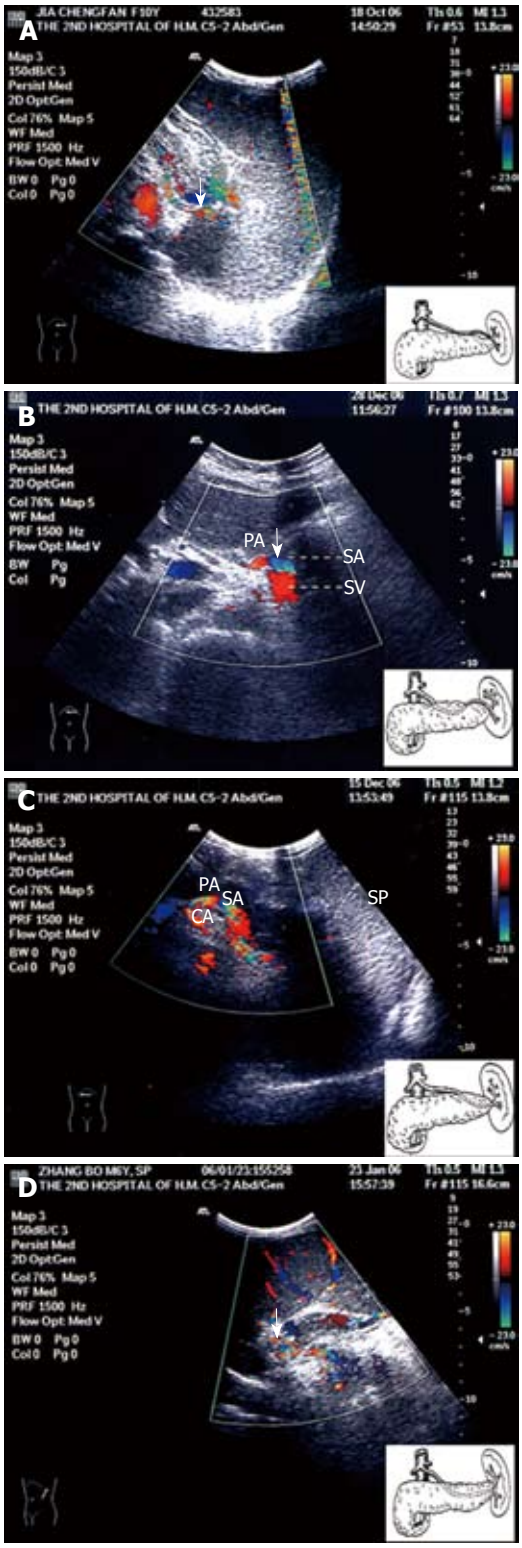

Figure 1 Sonogram and conceptual diagram of relationships between spleen vessels and pancreas. A: All spleen artery segments were located at the antero-superior border of pancreas (Type Ⅰ); B: One-fourth of the distal spleen artery segments were located at the antero-superior border of pancreas (Type Ⅱ); C: Two-fourths of the distal spleen artery segments were located behind or in the pancreas (Type Ⅲ); D: Three-fourths of the distal spleen artery segments were located behind or in the pancreas (Type Ⅳ).

diagnosis, CDFI can display 2-dimentional blood stream images directly and quickly[7,8]. CDFI has been used to prospectively assess resectable carcinoma in the head of

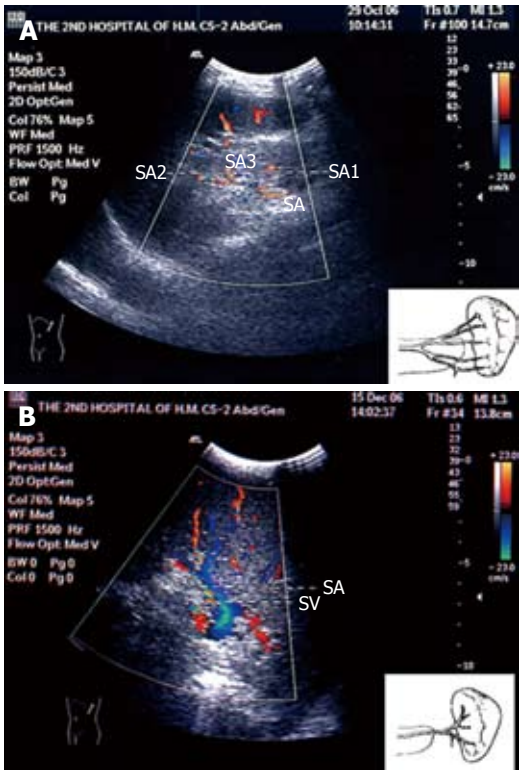

Figure 2 Sonogram and conceptual diagram of the spleen pedicle showing three isolated branches of spleen artery (A) (the distributed type) and the main stem of transparent spleen vessels (B) (the magisterial type).

pancreas and peri-ampulla since it can detect the tumor and blood vessels around it[9-11].

The spleen artery originates from the celiac artery and is divided into a segment upon the pancreas, a segment in the pancreas, a segment in front of the pancreas and a segment before the hilum of the spleen. One-fourth of the sub-terminal spleen artery segment keeps away from the pancreas. On the contrary, three-fourths of the distal pancreatic and spleen vessel segments are close to each[12-14]. In this study, the adjacent relations between spleen vessels and the pancreas were detected by preoperative CDFI. Four types of segment were found on CDFI. In type Ⅰ, three-fourths of the distal segments walked along the superior border of the pancreas constantly from celiac artery to hilum of spleen. In type Ⅱ, two-fourths of the middle segments were located behind or in the pancreas while one-fourth of the distal segments were located at the antero-superior border of the pancreas. In type Ⅲ, two-fourths of the distal segments walked behind or in the pancreas. In type Ⅳ, three-fourths of the distal segments were located behind or in the pancreas. Most adjacent relations belonged to type Ⅰ (approximately 45%), but the other types are consistent with those reported in the latest literature[13,14].

The anatomy of the spleen artery can be divided into magistral type and distributed type according to Michels' classification[15,16]. In this study, CDFI revealed that the anatomy of the spleen pedicle could also be divided into magistral type and distributed type. About 70% belonged to distributed type and 30% belonged to magistral type. The overall categorization accuracy

of spleen arterial geography was perfect according to Michels' classification.

Since hemorrhage is the major reason of conversion during LS, which is closely related to trauma of spleen pedicle and segment[17-20], operation can be performed safely and effectively if the surgeon fully understands the anatomic information of the spleen vessels[21]. In this study, the anatomic types of spleen pedicle and the adjacent relations between spleen vessels and pancreas were detected by preoperative CDFI and compared with the actual anatomy during LS. The results showed that their constituent ratio had no significant statistical difference and the coincidence rate almost reached 92% (23/25), suggesting that preoperative CDFI provides significant anatomic information of the spleen, thus operative procedures can be performed for type I and type Ⅱ spleen artery segments, the trunk of spleen artery can be ligated to control intra-operative hemorrhage and megalosplenia[5,22-24]. For type Ⅲ and type Ⅳ spleen artery segments, surgeons should elevate the anus perineum of spleen, separate anadesma tissue around the pancreatic tail and reveal the spleen pedicle.

In regard to the anatomic types of spleen pedicle, CDFI may lead surgeons to hilar approach strategy, particularly during the learning curve time. Surgeons may use the vascular stapler to ligate the trunk of spleen pedicle directly when a magisterial type of vascularization is present, and use clips to separate and ligate the secondary vessels of spleen pedicle when the distributed type is present, which prevents damage to the pancreatic tail and decreases the incidence rate of spleen fever[25]. In addition, surgeons can judge the trunk or the branches of spleen vessels to be ligated according to the diameters of spleen vessels shown by CDFI.

In our study, CDFI was limited to the body corresponded to the caudal extension of the spleen, which prevented a complete assessment of the remote spleen vessels.

Individual operation procedure can be formed according to the anatomic parameters of spleen provided by preoperative CDFI which can increase the safety and shorten intra-operative hemorrhage rate.

ACKNOWLEDGMENTS

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COMMENTS

Background

Laparoscopic splenectomy can be performed safely and effectively only when surgeons are familiar with the anatomy of spleen vessels. Color Doppler imaging plays an important role in location of target vessels. The value of color Doppler flow imaging (CDFI) for spleen micro-invasive operation was explored in this study.

Breakthroughs

Conversion often occurs during laparoscopic splenectomy which is closely related to trauma of pancreas and intra-operative hemorrhage. Since methods such as magnetic resonance angiography may result in additional traumas and heavy economic burden on the patients, they are often not used. However, color Doppler flow imaging (CDFI) can provide anatomic information of spleen vessels not leading to trauma, thus make laparoscopic splenectomy more safe and effective.

Applications

Individual operative procedures can be formed according to the anatomic parameters of spleen provided by preoperative CDFI. As a commonly used examination, CDFI may have an application prospect in micro-invasive surgery of spleen.

Terminology

CDFI is the latest diagnostic technique for the detection of heart and blood vessels without trauma by use of Doppler principle combined with B model imaging and M model echocardiogram. Furthermore, CDFI can display the direction and relative velocity of blood stream and provide temporal and spatial information of blood vessels.

Peer review

In this manuscript, the authors evaluated and classified the anatomy of spleen vessels into four types and two large categories based on the preoperative CDFI and compared the results with intra-operative recordings. The results are acceptable and helpful. The statistical analysis and all figures are adequate.

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CASE REPORT

Intestinal endometriosis-A rare cause of colonic perforation

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Abstract

Endometriosis is the ectopic growth of viable endometrium outside the uterus, affecting approximately 7% of females. It commonly affects pelvic structures including the bowel. Perforation of the colon by endometriosis is very rare and the patients generally present with an asymptomatic or painful pelvic mass, often in the left iliac fossa. Our patient presented acutely unwell and her symptoms were more suggestive of pyelonephritis or diverticulitis. We therefore report an unusual cause of acute abdomen. The purpose of the following case report is to elucidate certain diagnostic and therapeutic problems of the disease, concerning both surgeons and gynaecologists. In summary, intestinal endometriosis should be considered in the differential diagnosis of all post-menarche women with episodic gastrointestinal symptoms. A past history of endometriosis or co-existent gynaecological symptoms should increase the index of suspicion, and laparoscopy prior to formal laparotomy should be considered. Our patient, in retrospect, had a history of mild endometriosis, but we feel that this case serves as a reminder of a rare, but important, differential diagnosis of acute abdomen in females.

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Key words: Endometriosis; Colonic perforation; Intestinal endometriosis; Sigmoid colectomy; Sigmoid perforation

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INTRODUCTION

Endometriosis is the ectopic growth of viable endometrium outside the uterus, affecting approximately 7% of females. It commonly affects pelvic structures including the bowel. Perforation of the colon by endometriosis is very rare and the patients generally present with an asymptomatic or painful pelvic mass, often in the left iliac fossa. Our patient presented acutely unwell and her symptoms were more suggestive of pyelonephritis or diverticulitis. We therefore report an unusual cause of acute abdomen. The purpose of the following case report is to elucidate certain diagnostic and therapeutic problems of the disease, concerning both surgeons and gynaecologists.

CASE REPORT

The patient, a previously fit and well 44 years old female, presented with a 10 d history of worsening colicky pain in the left flank and iliac fossa. She had suffered form mild endometriosis in the past. On examination, she was pyrexial (39℃) with guarding over a mass in the left iliac fossa. Initial investigations revealed a white blood cell count of 22 000 per mm^3 and a urine dipstick weakly positive for blood, proteins, ketones and negative for beta-human chorionic gonadotropin. An urgent ultrasound suggested mild pelvicalyceal dilation on the left but a subsequent intravenous pyelography (IVP) was normal. A provisional diagnosis of a diverticular abscess was made and an emergency laparotomy was performed.

At surgery, a large endometriotic mass arising from the left fallopian tube and ovary, which had adhered to the sigmoid colon and then perforated it, was found. A left hemicolectomy with a proximal colostomy was performed and the endometriotic mass resected. The distal lumen of the bowel was closed with staples. Due to the large amount of peritoneal contamination, the wound was initially left open and underwent a delayed primary closure 9 d later. Postoperatively, the patient

made a steady recovery, initially requiring total parenteral nutrition. The distal part of her wound broke down and was allowed to heal by secondary intention. Colostomy was closed after three months and was referred to the gynaecologists for further management.

Following review by the gynaecologists, the patient was placed on the gonadotrophin releasing hormone agonist nafarelin, which is administered as a nasal spray, and placed on hormone replacement therapy with Premique [conjugated oestrogens (equine) 625 mg and medroxyprogesterone acetate 10 mg]. A combined procedure of hysterectomy and bilateral salpingo-oophrectomy (BSO) was planned for definitive management.

PATHOLOGY

The segment of excised sigmoid colon measured about 22 cm in length and up to 5 cm in external diameter. The colonic mucosa was oedematous with haemorrhagic mottling. An elongated fusiform cystic lesion or abscess was noted in the pericolic fat, measuring approximately 12.5 cm in length and 4 cm in transverse diameter, with a haemorrhagic lining. Approximately 6 cm away from this area was another cystic space, about 0.6 cm in diameter, which appeared to extend from the serosal surface of the specimen into at least the submucosa of the colon. Mucinous material was present within the latter area.

On histological examination, the smaller of the pericolic cavities was found to be an abscess communicating with the colonic lumen *via* a narrow channel. This was lined mainly by oedematous and acutely inflamed granulation tissue and degenerated endometriod type epithelium, together with macrophages filled with brown pigment, consistent with haemosiderin, scattered along the wall. Further areas of endometriotic glands and stroma were present in the adjacent pericolic fat. These features confirmed the clinical suspicion of a colonic perforation caused by endometriosis (Figure 1).

The section of the much larger pericolic abscess showed only acutely inflamed granulation tissue lining its cavity. Endometriotic tissue was not identifiable in the wall of this abscess, although, based on the features of the other abscess cavity, it is likely that also this larger abscess was the result of endometriosis. The overlaying colonic submucosa and mucosa were markedly oedematous and acutely inflamed. Extensive endometriosis was also present within the muscularis propria at the site of, and adjacent to, one of the colonic mucosal resection margins.

DISCUSSION

Endometriosis refers to extrauterine location and growth of endometrial tissue. Intestinal endometriosis occurs in 12% to 15% of cases, most often affecting those segments located within the pelvis, such as the terminal ileum, the appendix, the sigmoid colon and the rectum, both above and below the peritoneal reflection. The most common sites are the rectosigmoid (up to 73% of cases) and rectovaginal septum (13%). It predominantly involves the extra mucosal layers[1].

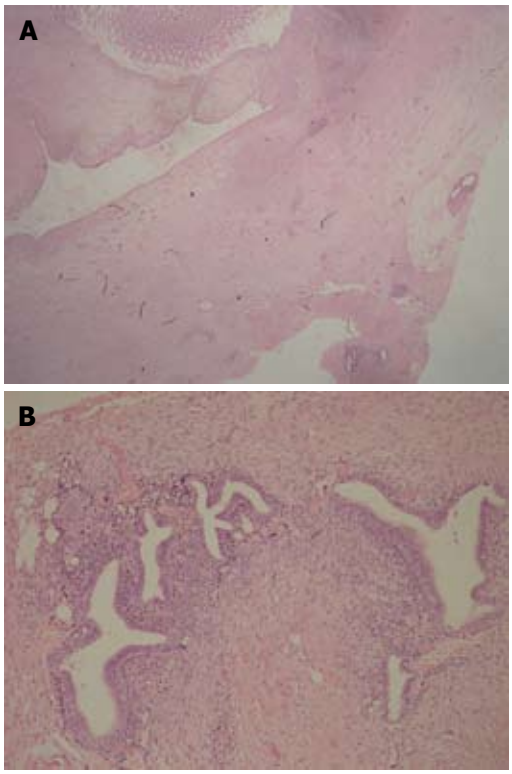

Figure 1 Histology findings. A: A low-power image of the colonic wall, with a few endometrial glands and stroma embedded in the muscular layer; B: A high-power view of the colonic wall, with endometrial glands and stroma embedded in the smooth muscle of the colon.

Intestinal endometriosis usually takes the form of asymptomatic, small and superficial serosal implants. However, under cyclical hormonal influences, these implants may proliferate and infiltrate the bowel wall. Cyclical haemorrhage from the endometrioma causes an intense localised fibrotic reaction in the bowel wall and the formation of strictures. Serosal involvement results in the formation of adhesions to neighbouring pelvic structures and bowel loops. This may result in intestinal obstruction with recurrent abdominal pain and alteration in bowel habits.

Endometriosis has also been reported in more distant locations, such as lungs, pleura and the umbilicus. Although the aetiology is uncertain, several hypotheses have been proposed concerning how the ectopic tissue reaches these locations, the most widely accepted being retrograde menstruation, although metaplastic change or metastatic spread may also occur[2].

Despite extensive serosal and intramural involvement, the intestinal mucosa usually remains intact and the endometrial perforation of the affected bowel is a very rare complication, which generally occurs in pregnant females. Haufler[3] first reported it in 1931 and, overall, we could find only seven cases of bowel perforation due to intestinal endometriosis in the literature. Six of them concerned pregnant women and in three cases the perforation involved their sigmoid colon.

Haufler[3] described a jejunal perforation due to

rupture of an endometriotic cyst during the sixth month of pregnancy in a 30-year old woman. In 1955, Henriksen[4] briefly mentioned a case of sigmoid perforation in his series of 1000 cases of endometriosis. Clement[5] in 1977, Rud[6] in 1979, and, most recently, Schweitzer[7] also reported similar cases of sigmoid colonic perforation secondary to endometriosis during pregnancy. Gini *et al*[8] in 1981, reported a case of appendiceal rupture through endometriotic tissue during the 35th wk of gestation. Most recently, Floberg *et al*[9] reported a 41-wk pregnant woman who perforated an endometriotic area of the sigmoid colon immediately postpartum.

A review of these reports reveals that most of the bowel wall, particularly the muscular layers, was replaced by endometriotic tissue. The most commonly reported symptom was intermittent crampy abdominal pain. These symptoms may not coincide with the menstrual cycle. McArthur and Ulfelder[10] observed that the area of endometrium had become decidualized and enlarged during the first trimester, and had undergone decidual necrosis with contraction during the third trimester. After pregnancy, there usually was a continued reduction in the size of the endometriotic lesions. This shrinkage may weaken the affected tissues and lead to rupture, particularly in the third trimester. This corresponds to the time of rupture in most of the previously reported cases.

Our present case differs from the previous reports in that the perforation occurred in a nonpregnant woman.

The treatment of uncomplicated intestinal endometriosis depends on the patient's age and intention to conceive. Bowel resection is indicated if there are symptoms of obstruction or bleeding, and if malignancy cannot be excluded. In patients of child-bearing age, resection of the involved colon followed by hormonal treatment may be sufficient; otherwise, hysterectomy and bilateral oophorectomy is the treatment of choice.

In summary, intestinal endometriosis should be considered in the differential diagnosis of all post-menarche women with episodic gastrointestinal symptoms. A past history of endometriosis or co-existent gynaecological symptoms should increase the index of suspicion, and laparoscopy prior to formal laparotomy should be considered. Our patient, in retrospect, had a history of mild endometriosis, but we feel this case serves as a reminder for a rare but important differential diagnosis of acute abdomen in females.

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CASE REPORT

Primary localized malignant biphasic mesothelioma of the liver in a patient with asbestosis

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Abstract

We report a case of primary localized malignant biphasic mesothelioma of the liver in a 66-year-old man associated with asbestosis. The tumor was detected as a hepatic nodule, 4 cm in diameter, in the right lobe (S8 segment) on CT scan. Histopathological examination demonstrated an intrahepatic tumor with central necrosis consisting of a papillary epithelioid pattern on the surface of the liver, microcystic (microglandular or adenomatoid) pattern mainly in the subcapsular area and sarcomatoid pattern intermingled with microcystic pattern in the major part of the hepatic nodular tumor. Tumor cells, especially of epithelioid type, showed distinct immunoreactivity for mesothelial markers (WT-1, calretinin, D2-40, CK5/6, mesothelin, thrombomodulin) and no immunoreactivity for epithelial (adenocarcinoma) markers (CEA, CD15, BerEP4, BG8, MOC31). P53 immunoreactivity was detected focally in papillary epithelioid tumor cells and extensively in microcystic and sarcomatoid components, suggesting that the papillary epithelioid mesothelioma arose on the surface of the liver, and tumor cells showing microcystic and sarcomatoid patterns invaded and grew into the liver. To date, this is the first case of primary localized malignant biphasic mesothelioma of the liver, since all three primary hepatic mesotheliomas reported so far were epithelioid type.

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Key words: Malignant mesothelioma; Pulmonary asbestosis; Liver; Immunohistochemistry

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INTRODUCTION

Malignant mesothelioma is a rare neoplasm of mesothelial cells arising most frequently in the parietal or visceral pleura and much less commonly in the peritoneum and pericardium[1-3]. In about 80%-90% of these patients, malignant mesothelioma is related to occupational exposure to asbestos in the air. The latent development period is long, about 25-40 years after initial exposure[3]. The disease is more common in men with a mean age of 58 years, with a male to female ratio of 4:1[1,4]. Mesothelioma occurring at various sites other than pleura, peritoneum and pericardium has been reported previously[4-7]. The mesothelial nature of the lesions is supported by immunohistochemical or electron microscopic evidence[3]. Most malignant mesotheliomas grow widely over the serosal membrane surfaces and tumors in the later stages eventually encase organs surrounding the involved site. Diffuse malignant mesothelioma typically has a poor clinical course with death occurring in most patients within 2 years of diagnosis[3]. In contrast, Allen *et al*[8] have recently reported a series of localized malignant mesotheliomas as uncommon sharply circumscribed tumors of the serosal membranes with the microscopic appearance of

diffuse malignant mesothelioma. They proposed that localized malignant mesotheliomas should be separated from diffuse malignant mesotheliomas because of their localized presentation, quite different biological behavior, and far better prognosis[8].

Primary malignant mesothelioma arising in the liver is very rare and there have been only 3 adult cases in previous reports[9-12], to our knowledge. In addition, there is one reported case of malignant cystic mesothelioma in an infant[11]. Although several primary hepatic tumors termed "localized fibrous mesotheliomas" have been reported[2,13-17], this type of tumor is better classified as a solitary fibrous tumor rather than mesothelioma retrospectively, because of the characteristic CD34 immunoreactivity[16] and limited evidence of a mesothelial nature[18].

In this report, we describe a case of primary localized malignant biphasic mesothelioma of the liver showing a nodular hepatic tumor and histologically sarcomatoid and epithelioid patterns in a 66-year-old man associated with asbestosis, and review the literature on primary malignant mesothelioma of the liver.

CASE REPORT

Clinical Findings

The patient (66-year-old man) had been followed up for hypertension and pulmonary asbestosis caused by occupational exposure to asbestos. At the age of 66, CT revealed a hepatic nodule, 4 cm in diameter, in the S8 segment of the liver (Figure 1) on periodic examination for pulmonary asbestosis. The hepatic nodule was located just under the diaphragm, wedge-shaped and slightly low density compared to the surrounding hepatic parenchyma on plain CT. Peripheral staining of the hepatic nodule was seen on enhanced CT (Figure 1). Cholangiocarcinoma or inflammatory pseudotumor was suspected from CT findings. The findings signifying pulmonary asbestosis showed on chest X-ray and chest CT as previously detected, but there were no new lesions suggesting lung cancer or pleural mesothelioma (Figure 1). There was no finding suggesting peritoneal mesothelioma or a primary mesothelioma of the tunica vaginalis testis. Examination using [^{18}F]-fluoro-deoxy-D-glucose positron emission tomography (FDG-PET) revealed no additional lesions other than the hepatic tumor. At that time, the serum levels of ALT, AST, Al-P, r-GTP and LDH were within the normal range. AFP, CEA and CA19-9 were negative. Viral markers related to hepatitis B virus (HBV) and hepatitis C virus (HCV) infection were negative. The patient was not a drinker. The needle biopsy revealed that the hepatic tumor was a sarcomatoid malignant tumor with immunoreactivity for cytokeratin and vimentin, and with faint and focal immunoreactivity for calretinin and D2-40. The papillary and microcystic components were not included in the biopsy. The patient underwent a hepatic segmentectomy (S8). A few sclerotic nodules attached to the diaphragm were also removed during the operation. Recurrence and metastasis of the tumor have not been detected by 6 mo after the operation.

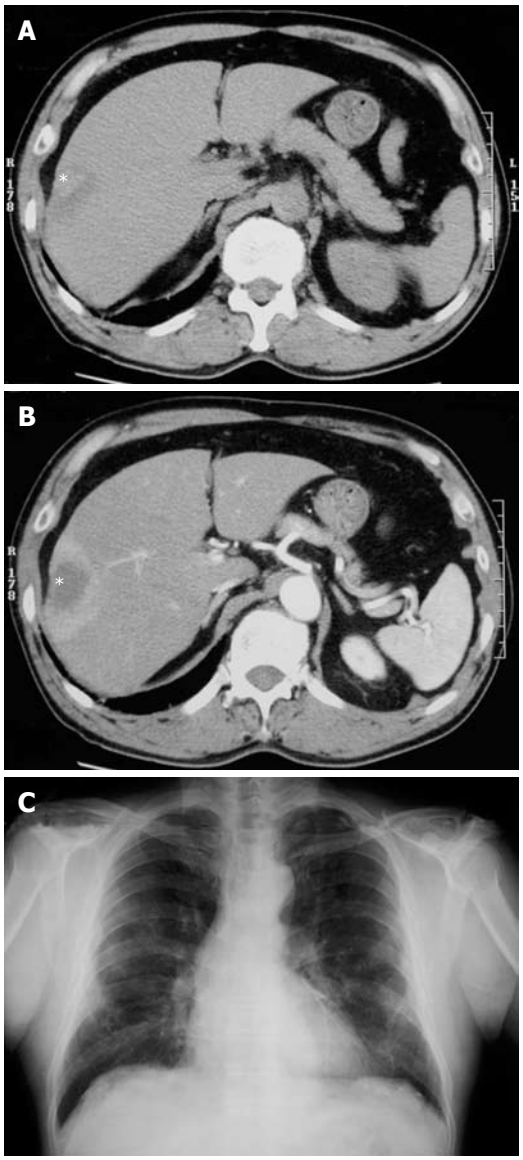

Figure 1 Radiological findings. A: Plain CT showed a low-density mass (asterisk) in S8 segment of the liver; B: The periphery of the mass (asterisk) was enhanced in the early phase of enhanced CT, suggesting abundant blood supply; C: Chest X-ray showed pulmonary asbestosis and pleural thickening, but there were no new lesions suggesting lung cancer or pleural mesothelioma.

Gross pathology examination

The resected specimen consisted of a 12.8 cm × 17 cm × 7 cm portion of the liver (445 g). On the surface faced to the diaphragm, a firm, white and slightly depressed lesion measuring 4.2 cm × 3 cm was identified (Figure 2). On sectioning of the liver, a yellowish-white tumor of 4.4 cm × 3.8 cm was identified just under the hepatic capsule. The tumor had well-defined borders and contained central necrosis (Figure 2). The tumor mainly grew within the hepatic parenchyma, not in the surface of liver, and therefore adhesion did not occur between the diaphragm and hepatic capsule. There were no daughter nodules in the liver and the surgical margin was free from tumor. The background liver appeared normal macroscopically.

Histologic findings

Hematoxylin and eosin stained sections showed a

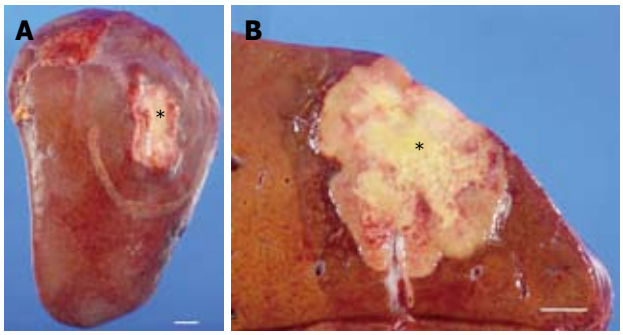

Figure 2 Gross appearance of the tumor. A: A firm, white and slightly depressed lesion (asterisk) measuring 4.2 cm × 3 cm was identified on the liver surface. B: Cross section of the tumor in the liver. A yellowish-white tumor of 4.4 cm × 3.8 cm was identified just under the hepatic capsule (asterisk). The tumor had well-defined borders and an area of central necrosis. Bar indicates 1 cm.

tumor composed of three main components: (1) sarcomatoid, (2) papillary epithelioid and (3) microcystic (microglandular or adenomatoid) (Figure 3). The sarcomatoid component was predominant and was composed of tumor cells showing trabecular structures separated by bands of fibrous tissue (Figure 3A). The tumor cells had abundant eosinophilic or clear cytoplasm, indistinct cytoplasmic borders, round and atypical nuclei with vesicular fine chromatin and variably sized nucleoli (Figure 3A). There were many mitoses and atypical mitoses in tumor cells. The tumor contained broad coagulation necrosis. Although the tumor had well-defined borders, there was no fibrous capsule and hepatocytes and bile ducts were entrapped in infiltrating tumor cells. In the white lesion on the surface, papillary proliferation of epithelioid tumor cells was identified. Epithelioid cells had eosinophilic cytoplasm with bland nuclei and distinct nucleoli. Near the surface, tumor cells showed microcystic structures with a lace-like, adenoid cystic or signet ring appearance (microcystic, microglandular or adenomatoid component). The microcystic component was located adjacent to the papillary epithelioid component (Figure 3B). Furthermore, microcystic component was also intermingled with the sarcomatoid component even in the deeper area adjacent to the surrounding hepatic parenchyma (Figure 3C). The background liver showed mild steatofibrosis and there was no intrahepatic metastasis of the tumor. The biopsy site could not be identified by gross and histological examination in the surgical specimen.

A few sclerotic nodules attached to the diaphragm were composed of dense hyalinized fibrous tissue resembling pleural plaques. There were no tumor cells in the nodules.

Immunohistochemical and special stains

Formalin-fixed, paraffin-embedded sections were stained with periodic acid-Schiff (PAS) with and without diastase digestion, and alcian blue (pH 2.5) with and without hyaluronidase digestion. Immunohistochemical stains were performed using the Envision methods

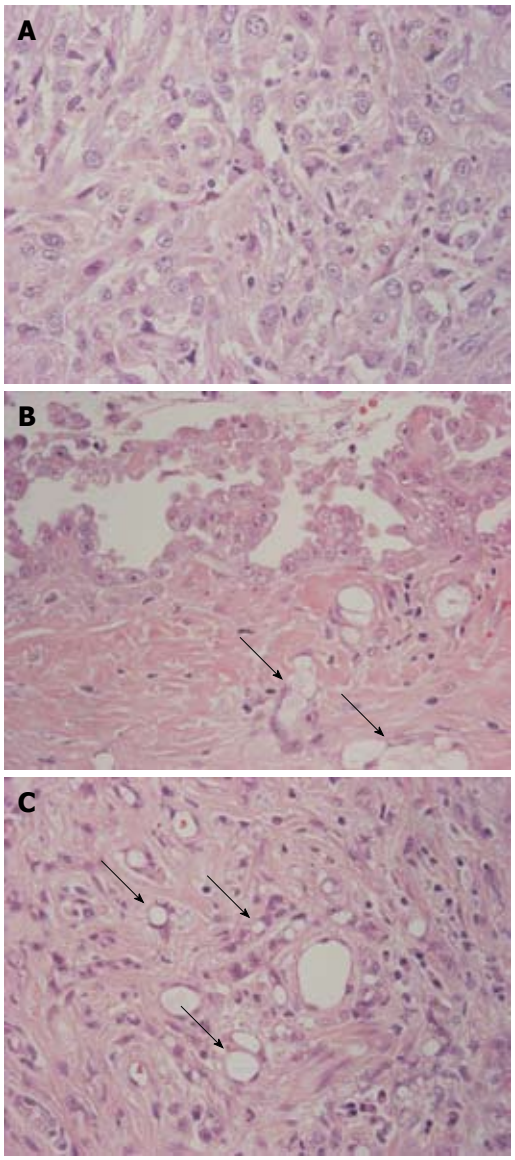

Figure 3 Histologic features of the tumor. A: Sarcomatoid component was predominant and was composed of tumor cells with abundant eosinophilic or clear cytoplasm, indistinct cytoplasmic borders, round and atypical nuclei with vesicular fine chromatin and variably sized nucleoli; B: Papillary proliferation of epithelioid tumor cells was identified on the surface of the tumor. Epithelioid cells had eosinophilic cytoplasm with bland nuclei and distinct nucleoli. Arrows indicate microcystic (microglandular or adenomatoid) component; C: In the area near the surface, a microcystic (microglandular or adenomatoid) component showing microcystic structures with lace-like, adenoid cystic or signet ring appearance was detected (arrows) (HE × 400).

(Dako, Carpinteria, CA, USA) and antibodies as described previously[19]. The antibodies included WT-1 (6F-H2, Dako), calretinin (polyclonal; no dilution; Nichirei, Tokyo, Japan), D2-40 (D2-40; dilution 1/100; Dako), CK5/6 (D5/16B4, dilution 1/50; Dako), mesothelin (5B2, dilution 1/20; Lab vision, Fremont, CA, USA), thrombomodulin (1009, dilution 1/20; Dako), vimentin (3β4, dilution 1/600; Dako), CD34 (Immu133, dilution 1/200; Immunotech, Fullerton, CA, USA), p53 (DO-7; dilution 1/100; Dako), epithelial membrane antigen (EMA) (E29; dilution 1/200; Dako), MUC1 (DF3, Toray-Fuji Bionichs, Tokyo, Japan), Ber-EP4 (Ber-EP4; no dilution; Neomarkers, Fremont, CA,

USA), cytokeratin (polyclonal; dilution 1/600; Dako), MOC-31 (MOC-31; dilution 1/50; Dako), CK7 (OV-TL 12/30; dilution 1/50; Dako), CK19 (RCK108; dilution 1/50; Dako), CEA (II-7, dilution 1/100, Dako), CA19-9 (C241:5:1:4, dilution 1/100; Novocastra, Newcastle, UK), CD15 (C3D-1; dilution 1/50; Dako), BG-8 (F-3; dilution 1/200, Signet, Dadham, MA, USA), HepPar1 (OCH1E5, dilution 1/100; Dako). Heat-induced epitope retrieval was used for all antibodies except CD34, MUC1, CA19-9 and BG-8. Appropriate positive and negative controls were used throughout.

Alcian blue showed positive staining on the surface of papillary epithelioid cells and the lumen of microcystic component, and this positive staining was sensitive to digestion with hyaluronidase. Neutral mucin stained by PAS stain was not detected on the surface of papillary epithelioid cells or the lumen of the microcystic component. Immunohistochemical stains confirmed the mesothelial features of the tumor showing membranous D2-40, mesothelin and thrombomodulin and nuclear WT-1, in addition to nuclear and cytoplasmic calretinin immunoreactivity, mainly in the papillary epithelioid and microcystic components (Figure 4). Sarcomatoid tumor cells showed focal immunoreactivity for WT-1, D2-40 and calretinin. When we examined the immunoreactivity for D2-40 and calretinin in 23 intrahepatic cholangiocarcinoma samples for comparison, none of the cholangiocarcinoma showed nuclear immunoreactivity for calretinin or immunoreactivity for D2-40. Four cholangiocarcinomas showed weak cytoplasmic immunoreactivity for calretinin. The tumor cells showed focal immunoreactivity for CK5/6. The tumor cells showed strong immunoreactivity for vimentin, CK7, CK19 and polyclonal cytokeratin (Figure 4). Strong EMA and MUC1 immunoreactivity was seen on the surface of papillary epithelioid cells, the lumen of the microcystic component and part of the sarcomatoid component (Figure 4). Sarcomatoid and microcystic components showed strong nuclear immunoreactivity for p53 (Figure 4). A few positive cells were also seen in the papillary epithelioid component. The tumors showed no immunoreactivity for CEA, Ber-EP4, MOC-31, CA19-9, CD15, BG-8, CD34 and HepPar1.

DISCUSSION

Primary malignant mesothelioma arising in the liver is rare and is currently not listed in the World Health Organization classification of hepatic tumors[20]. Review of the literature disclosed only three previously reported adult cases of primary malignant mesothelioma[9,10,12]. The previously reported cases were two men[9,10] and a woman[12], ranging in age from 54 to 64 years old (mean: 60 years old). Two patients had no history of asbestos exposure[9,10] and there was no indication in one patient[12]. One case was associated with cirrhosis due to hepatitis C viral infection[9]. All tumors arose in the right hepatic lobe, located in the subcapsular area of the liver and were 3.2 to 12 cm in diameter (mean: 7 cm).

Histologically malignant mesotheliomas conform to one of three patterns: epithelial (the most common type), sarcomatoid and biphasic (mixture of epithelioid and sarcomatoid) types[1,3,4]. All three reported mesotheliomas arising in the liver were of the epithelioid type[9,10,12]. One tumor was composed of sheets of cytologically bland polygonal cells containing multiple gland-like spaces and microcysts lined by cuboidal to columnar epithelium[12]. The other tumors displayed the tubular[9,10] and papillary proliferation[10] of epithelioid cells with a desmoplastic stroma[9] or surrounded by a densely mixed inflammatory infiltrate[10]. A panel of immunohistochemical markers was applied for differential diagnosis in all three tumors as discussed below and electron microscopic findings suggested a mesothelial cell origin in two tumors[10,12]. One tumor was speculated to originate from mesothelial cells of Glisson's capsule[12]. However, no tumors were exposed to the peritoneal cavity on the hepatic capsule[9,10,12] and were thought to be localized mesothelioma of the liver, not to be localized mesothelioma of the peritoneum with hepatic invasion[9,10,12].

In this report, we have described for the first time a malignant mesothelioma of biphasic type arising in the liver in a 66-year-old man with a history of asbestos exposure. The tumor was detected as a hepatic nodular lesion in a follow-up examination for asbestosis and there was no other lesion suggesting primary tumor in the pleura and peritoneum. The tumor arose in the right lobe (S8 segment) of the liver in accordance with the reported cases[9,10,12]. In the major part of the hepatic nodular tumor, the tumor cells showed a sarcomatoid pattern and a microcystic pattern was intermingled with the sarcomatoid pattern. Tumor cells showed a papillary epithelioid pattern on the surface of the liver and a microcystic pattern was also seen in the subcapsular area near the surface. Since these tumor cells show the profile of mesothelioma even in the deeper area, this tumor is diagnosed as a malignant mesothelioma, not as another type of tumor with reactive mesothelial hyperplasia. The tumor in the present case may be categorized into the entity of localized malignant mesothelioma because of localized presentation[8], and better prognosis may be expected in the patient.

The origin of tumor cells showing mesothelial features in the liver is not clear, since mesothelial cells are not present in livers under normal physiological conditions. The mesothelioma cells might originate from other types by transition, although there has been no evidence of transition to mesothelial cells, so far. In contrast to the reported cases in which tumor cells did not expose to the peritoneal cavity, a papillary epithelioid component was also seen on the surface of the hepatic capsule adjacent to the hepatic nodular tumor. This finding suggests that the present tumor may originate from mesothelial cells of the Glisson's capsule which subsequently invaded into the liver. A reported tumor was also speculated to originate from mesothelial cells of the Glisson's capsule, although there was no evidence[10]. From a standpoint of p53 immunoreactivity, a few

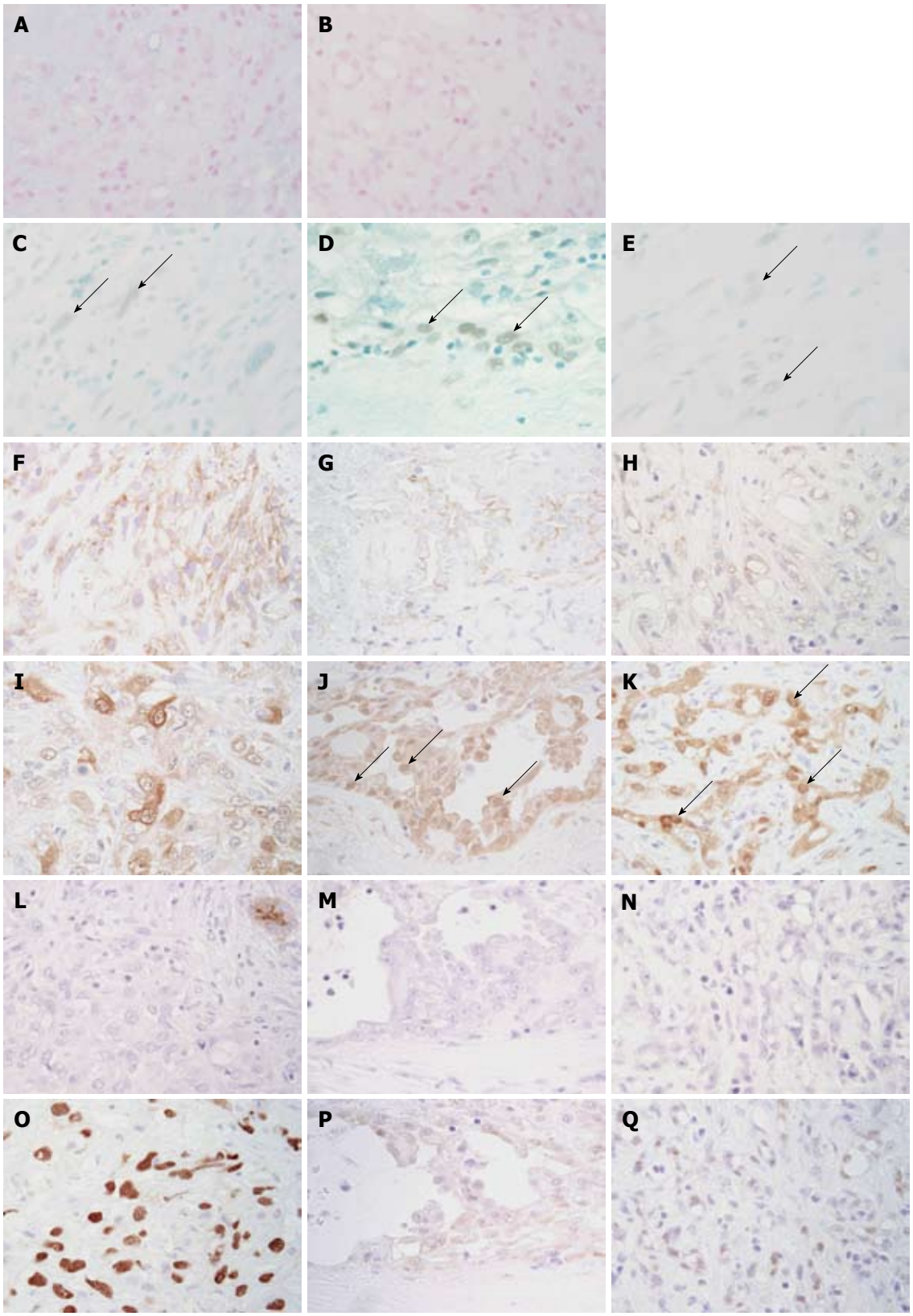

Figure 4 Immunohistochemical and special stains. Alcian blue (pH 2.5) showed positive staining on the lumen of adenomatoid tumors (A). This positive staining was sensitive to digestion with hyaluronidase (B). Nuclear WT-1 immunoreactivity (arrows) was detected focally in sarcomatoid (C), papillary epithelioid (D) and microcystic (E) components. Immunostaining for WT-1 and methyl green. Sarcomatoid tumor cells showed focal membranous immunoreactivity for D2-40 (F). Membranous D2-40 immunoreactivity was detected in the papillary epithelioid (G) and microcystic (H) components. Immunostaining for D2-40 and hematoxylin. Sarcomatoid tumor cells showed focal and rather weak immunoreactivity for calretinin (I). Nuclear calretinin immunoreactivity (arrows) was detected in the papillary epithelioid (J) and microcystic (K) components. Immunostaining for calretinin and hematoxylin. Immunoreactivity for CA19-9 was not detected in sarcomatoid (L), papillary epithelioid (M) and microcystic (N) components. The apical surface of the entrapped bile duct showed immunoreactivity for CA19-9 (L, upper right corner). Immunostaining for CA19-9 and hematoxylin. Sarcomatoid (O) and microcystic (P) components showed strong nuclear immunoreactivity for p53. A few papillary epithelioid cells showed nuclear immunoreactivity for p53 (Q). (Immunostaining for p53 and hematoxylin, × 400).

cells showed faint p53 immunoreactivity in papillary epithelioid components, whereas most tumor cells showed strong p53 immunoreacitivity in microcystic and sarcomatoid components. Therefore, it is conceivable that the mesothelioma of papillary epithelioid type initially arose on the surface of the liver and tumor cells showing microcystic and sarcomatoid patterns invaded and grew into the liver with more aggressive features originating from p53 mutation in the present case. Since this tumor was located on the hepatic capsule just under the diaphragm and compressed, it may be easier for tumor cells to grow into hepatic parenchyma than spreading in the surface of the liver, and therefore adhesion did not occur between the diaphragm and hepatic capsule. Taken together, a localized mesothelioma of the liver may arise as a localized mesothelioma of the Glisson's capsule (peritoneum) and may subsequently show an intrahepatic nodular growth.

The differential diagnosis of primary malignant mesothelioma of the liver includes primary liver cancers and metastases from pleural malignant mesothelioma. In particular, it is important but may be difficult to distinguish from intrahepatic cholangiocarcinoma. Since both malignant mesothelioma of epithelioid type and cholangiocarcinoma show a tubular and papillary structure[3,20], a similar panel of immunohistochemical markers may be useful in the differential diagnosis between pleural mesothelioma and pulmonary adenocarcinoma, which is sometimes difficult[3,21-23]. There is no single absolute marker for mesothelioma, so far[3]; therefore, a combination of two or more positive immunohistochemical mesothelial markers (CK5/6, calretinin and Wilms tumor gene-1 (WT1)) with negative epithelial (adenocarcinoma) markers (CEA, CD15, BerEP4, B72.3, BG8, MOC31) is recommended for a diagnosis of pleural mesothelioma[3,21,23]. Since both malignant mesothelioma and cholangiocarcinoma show immunoreacitivity for CK7, CK19, polyclonal cytokeratin, EMA (a glycosylated form of MUC1) and unglycosylated form of MUC1 detected by DF3, these markers are not helpful for differential diagnosis. Recently, D2-40 was reported to be a sensitive marker for cells of mesothelial origin, and useful in the differential diagnosis of epithelioid malignant mesothelioma vs adenocarcinoma[22]. In previous reports[9,10,12], the diagnosis of primary hepatic mesotheliomas has been made based on positive immunoreactivity for calretinin[9,10,12], HBME-1[9], D2-40[10], thrombomodulin[10], vimentin[10,12] and cytokeratins[9,10,12] and negative immunoreactivity for CEA[9,10], LeuM1 (Lewis X)[9], CD34[9,10,12], CA19-9[10] and Ber-EP4[10]. There have been no reports regarding immunoreactivity for calretinin and D2-40 in intrahepatic cholangiocarcinoma, so far. In our preliminary study, cytoplasmic, but not nuclear calretinin immunoreactivity was detected in some intrahepatic cholangiocarcinomas and D2-40 imunoreactivity was not detected in any intrahepatic cholangiocarcinomas examined. Since the expression of CEA and LeuM1 (Lewis X) is less frequent in poorly differentiated intrahepatic cholangiocarcinoma[24] and the specificity of HBME-1 for mesothelioma is rather low (45%)[21], a combination of calretinin, HBME-1, CEA and LeuM1 may not be enough for a definite diagnosis of mesothelioma in the previously reported primary hepatic mesothelioma arising in a patient with cirrhosis and chronic hepatitis C[9]. The possibility of cholangiocarcinoma associated with cirrhosis and chronic hepatitis C[25] should be excluded in the reported case[9].

In the present case, tumor cells, especially of epithelioid type, showed distinct immunoreactivity for mesothelial markers (WT-1, calretinin, D2-40, mesothelin, thrombomodulin) and no immunoreactivity for epithelial (adenocarcinoma) markers (CEA, CD15, BerEP4, BG8, MOC31). Markers suggesting other types of tumor such as CD34, CD31, HepPar1 were totally negative; therefore the tumor fulfills the immunohistochemical diagnostic criteria for mesothelioma. However, it was quite difficult to reach a final diagnosis from a needle biopsy specimen including only the sarcomatoid component with immunoreactivity for cytokeratin and vimentin, and faint and focal immunoreactivity for calretinin and D2-40. It is reported that intrahepatic cholangiocarcinoma shows only occasional sarcomatous change[20,26]. In this type, tumor cells show immunoreactivity for both epithelial markers (cytokeratins and EMA) and vimentin[20,26]. Clinical information about asbestos exposure was important to raise the possibility of mesothelioma.

In summary, we presented for the first time a localized malignant biphasic mesothelioma arising in the liver in a 66-year-old man with a history of asbestos exposure. A panel of immunohistochemical markers to distinguish pleural mesothelioma from pulmonary mesothelioma was useful for diagnosis. Malignant mesothelioma should be included in the differential diagnosis of primary hepatic tumor, especially in patients with a history of asbestosis exposure. Because of unusual localization of the tumor, a very careful histological and immunohistochemical examination was required to reach the final diagnosis in the present case. Accumulation of more cases similar to the present case is important to characterize the features of mesothelioma of the liver, including the biological behavior and the prognosis.

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CASE REPORT

A variant form of autoimmune pancreatitis successfully treated by steroid therapy, accompanied by von Meyenburg complex

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Abstract

Diagnostic criteria for autoimmune pancreatitis (AIP) have been proposed and used clinically because, despite its unique clinicopathological features, AIP does not have disease-specific serological tests for confirmation. However, diagnosis of a patient with pancreatic lesions mimicking cancer who deviates from these diagnostic criteria is still difficult. We present herein a patient with a variant form of AIP successfully diagnosed by fine-needle biopsy, whose response to steroid therapy was excellent. A 55-year-old Japanese man was admitted to hospital because of jaundice and pancreatic head mass. AIP was considered as one of the differential diagnoses; however, as the patient showed neither pancreatic duct narrowing nor immunological abnormalities, he did not meet the Japanese diagnostic criteria for AIP. Histopathology of the pancreatic mass demonstrated abundant infiltration by lymphocytes and interstitial fibrosis, which suggested AIP. Immunoreaction to IgG4, which is supposed to be specific to AIP, was not observed; however, response to subsequent prednisolone therapy was good, with dramatic pancreatic head mass regression. Aside from the pancreatic head mass, diffusely spreading small lesions were observed throughout the liver. The likelihood of a potential association with extrapancreatic lesions of AIP was considered and led us to carry out a liver biopsy, which revealed biliary hamartoma, also called von Meyenburg complex (VMC). As IgG4-positive plasma cell infiltration was not demonstrated in the hamartomatous regions, the hepatic condition was thought to have occurred incidentally; however, to the best of our knowledge, this is the first report in which the association between AIP and VMC was investigated and discussed.

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Key words: Autoimmune pancreatitis; Diagnostic criteria; IgG4; Biliary hamartoma; von Meyenburg complex

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INTRODUCTION

Clinically unique chronic pancreatitis, in which autoimmune mechanisms are supposed to be involved, has been focused upon and investigated intensively over the last decade[1-3], and is now recognized as a clinical entity worldwide, autoimmune pancreatitis (AIP)[4-6]. Since disease-specific serological tests for determining AIP remain unavailable, diagnostic criteria for AIP have been proposed by incorporating characteristic clinical aspects of the disease[7-11]. Japanese criteria were established first in the world in 2002 by the Japan Pancreas Society (JPS)[7]; however, with the accumulation of clinical investigations of AIP, the JPS criteria were modified in 2006 by the Research Committee of Intractable Pancreatic Diseases (RCIPD)[9], because the concept of AIP has changed gradually with the detection of additional new clinicopathological aspects,

such as a high concentration of serum IgG4[12], a variety of extrapancreatic involvement relevant to IgG4-positive lymphoplasmacytic infiltration[13-15], and morphologically atypical localized AIP[16-19]. On revision, these new clinical findings were included in the criteria, enabling more cases to be diagnosed. However, because Japanese criteria mandate first identification of characteristic imaging, namely, narrowing of the main pancreatic duct demonstrated by endoscopic retrograde cholangiopancreatography (ERCP) and enlargement of the pancreas, in order to exclude absolutely pancreatic or biliary cancer rather than picking-up suspicious AIP patients[9], some AIP patients remain undiagnosed as a result of being unmatched with the Japanese criteria for AIP. Yet, because AIP has a favorable prognosis if steroid therapy is administered appropriately[20], we should also pay attention to atypical forms of AIP before a patient undergoes an operative procedure.

We describe herein a case of the variant form of AIP diagnosed by fine-needle aspiration biopsy, and the subsequent successful response to steroid therapy, whose primary presentation was obstructive jaundice caused by the pancreatic head mass alone, without other specific features outlined in the Japanese diagnostic criteria for AIP. In addition, the patient was found to concomitantly have biliary hamartoma (von Meyenburg complex; VMC), the initial clinical appearance of which first led us to consider them as possible extrapancreatic lesions of AIP.

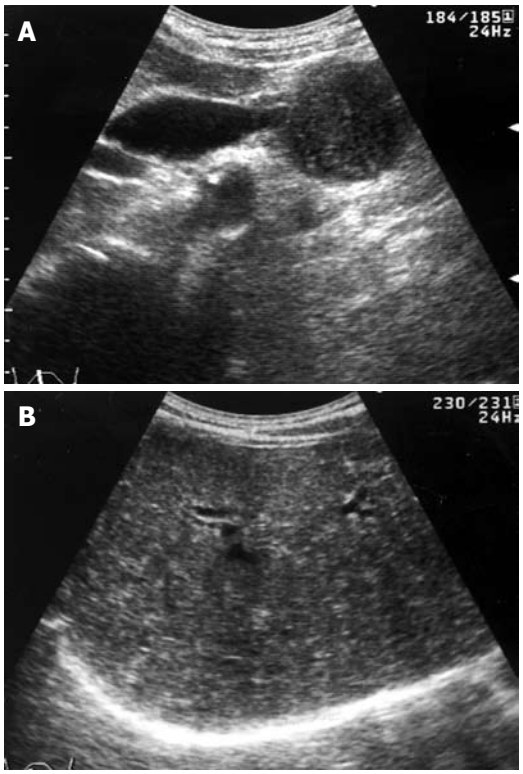

Figure 1 Abdominal US. A: Pancreatic head mass with hypo-echogenicity, 43 mm in diameter, which compressed the lower portion of the bile duct, and resulted in upstream bile tract dilatation; B: Many tiny, diffuse hyperechoic patches or comet-like tails that suggested the presence of certain intrahepatic abnormalities, but the reason could not be elucidated.

CASE REPORT

A 55-year-old Japanese man was referred and admitted to our hospital for the evaluation of jaundice and a pancreatic head mass on 2 May 2007. One week before admission, the patient had visited another hospital because of body weight loss and a 1-wk history of epigastralgia, and was found to have obstructive jaundice caused by pancreatic head swelling, upon abdominal ultrasongraphy (US). Upon admission, he was 170 cm tall and weighed 57.0 kg, blood pressure was 112/62 mmHg, and temperature was 37.1℃. There was no history of drug or alcohol abuse.

Physical examination revealed no abnormal findings except for jaundice of the conjunctiva and skin. Laboratory findings on admission are shown in Table 1. Abdominal US showed obstructive jaundice caused by a hypo-echoic pancreatic head mass that measured 43 mm in diameter, which compressed the lower portion of the bile tract, and resulted in intra- and extra-hepatic dilatation of the bile duct. In addition, many irregular hyperechoic spots and comet-like tails were found to spread diffusely in the bilateral liver parenchyma (Figure 1). Upon dynamic contrast-enhanced computed tomography (CT), the pancreatic head tumor was slightly less enhanced than the other portion of the pancreas, together with irregular dilatation of the main pancreatic duct distal to the tumor, which suggested pancreas cancer. However, the distal area of the pancreas was not atrophied (Figure 2A and B). Magnetic resonance cholangiopancreatography (MRCP) performed subsequently demonstrated intensive stenosis of the pancreatic head portion and peripheral dilatation of the common bile duct, as well as the skipped stenotic lesions of the main pancreatic duct (Figure 3). Simultaneously, on MRCP imaging, numerous hyper-intense round nodules of small diameter, presumably correlating with the US imaging of the liver, were found to be diffusely scattered throughout the liver (Figure 3). We could not perform ERCP because of the lack of an expert ERCP endoscopist in the hospital. Upon MRCP, skipped stenotic lesions of the main pancreatic duct, which was suggestive of AIP was observed; however, irregular narrowing of the pancreatic duct seen in typical AIP was not observed. The levels of tumor markers, carbohydrate antigen (CA 19-9, DUPAN-2) and carcinoembryonic antigen (CEA), were normal. Immunologically, antinuclear antibody was absent and the serum level of total IgG as well as IgG4 was within the normal range (Table 1). As we could not obtain diagnostic confirmation from these sequential atypical clinical features of AIP or pancreatic cancer, we performed US-guided percutaneous aspiration biopsy using a 21G fine needle that targeted the pancreatic head mass on 15 May 2007. Histopathological examination demonstrated marked infiltration of pancreas parenchyma by mononuclear cells and fibrosis adjacent to the lesion, which suggested a diagnosis of AIP (Figure 4A). There were no malignant cells indicating

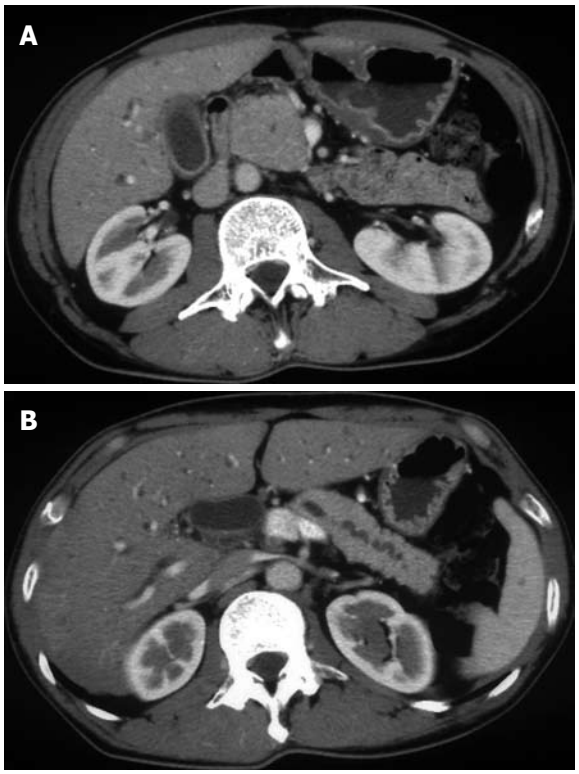

Figure 2 Abdominal enhanced CT imaging with contrast medium in May 2007. A: Swelling of the pancreatic head like a solid tumor was shown. B: The distal portion of the main pancreatic duct was found to be irregularly dilated; however, the pancreatic body and tail were not atrophied as seen in pancreatic cancer.

pancreatic cancer. These infiltrated cells were revealed to be mainly T lymphocytes by subsequent immunohistochemistry (Figure 4B), which was more compatible with AIP. However, plasma cell infiltration was scarce and immunoreaction to anti-IgG4 antibodies was not seen (Figure 4C).

The patient did not fulfill the JPS diagnostic criteria for AIP. However, as the presence of pancreatic cancer cells was histopathologically ruled out and the likelihood of spontaneous regression of obstructive jaundice seemed to be poor, we started oral prednisolone (PSL) therapy on 18 May 2007. Once PSL at an initial daily dose of 30 mg was introduced, prompt regression of the pancreatic head mass and improvement of bile and pancreatic duct dilatation was achieved (Figure 5A and B).

Apart from the pancreatic head mass, the presence of intrahepatic anomalies was suspected from the US and MRCP imaging. As AIP is known to show frequently a variety of extrapancreatic involvements, we carried out liver biopsy in order to elucidate the identity of the diffusely spreading aberrant echoes on US and small nodules seen on MRI, in addition to investigating the possible association with AIP. Histopathological examination of the hepatic parenchyma revealed aggregation of irregular-shaped cystic dilated bile ducts embedded in fibrous stroma, with minimal inflammatory reaction (Figure 6), and biliary hamartoma, also called VMC. Additionally, we performed immunohistochemistry using anti-IgG4 antibody, which resulted in negative interactions in those areas.

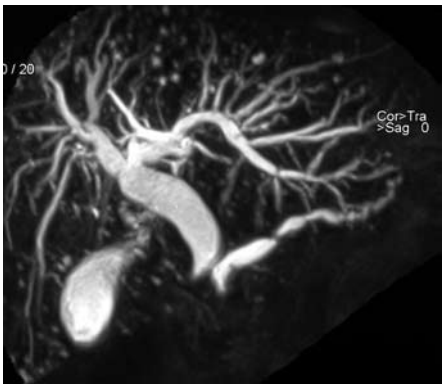

Figure 3 MRCP showed proximal stenosis of the bile duct as well as the main pancreatic duct; however, narrowing of the pancreatic duct, as seen in AIP, was not demonstrated at all. A simultaneous finding was that there were many round high-intensity nodules scattered throughout the liver.

Table 1 Laboratory data

	Normal value	May 2, 2007	July 14, 2007
WBC (White blood cell)	4000-9000/μL	10210	15350
RBC (Red blood cell)	410-530 × 10^4/μL	504	471
Hb (Hemoglobin)	14-18 g/dL	15.5	15.0
Plt (Platelet)	12-36 × 10^4/μL	33.9	23.4
TP (Total protein)	6.5-8.0 g/dL	6.8	6.9
Alb (Albumin)	3.9-4.9 g/dL	3.5	4.3
IgG (Immunoglobulin G)	870-1700 mg/dL	919	843
IgG4 (Immunoglobulin G4)	4.8-105 mg/dL	20.3	-
AST (Aspartate aminotransferase)	10-33 IU/L	49	19
ALT (Alanine aminotransferase)	4-30 IU/L	57	28
LDH (Lactate dehydrogenase)	100-230 IU/L	146	132
ALP (Alkaline phosphatase)	167-345 IU/L	992	265
γ-GTP (γ-glutamyl transpeptitase)	10-75 IU/L	140	51
TB (Total bilirubin)	0.2-1.2 mg/dL	4.6	0.7
DB (Direct bilirubin)	0-0.4 mg/dL	2.6	0.1
Amylase	30-120 IU/L	105	36
BUN (Blood urea nitrogen)	8-20 mg/dL	13	13
Cr (Creatinine)	0.6-1.1 mg/dL	0.6	0.7
CRP (C-reactive protein)	0-0.4 mg/dL	0.0	0.0

All laboratory abnormalities seen upon admission were normalized by 14 July 2007 (Table 1), and the patient remains in a good condition without recurrence at the present time, under maintenance therapy with 5 mg/d PSL.

DISCUSSION

This case report presents notable clinical information for dealing with AIP patients. First, the existence of a variant form of AIP not meeting the diagnostic criteria was verified by fine-needle aspiration biopsy, together with a subsequent dramatic good response to steroid therapy. Second, concomitant development of AIP and VMC was investigated and discussed for the first time, even though this condition might occur incidentally.

Sets of diagnostic criteria for AIP have been proposed by integrating several known unique morphological, immunological and histopathological characteristics of AIP. Currently, four major sets of diagnostic criteria for AIP have emerged from Japan[9], Italy[8], Korea[10] and the

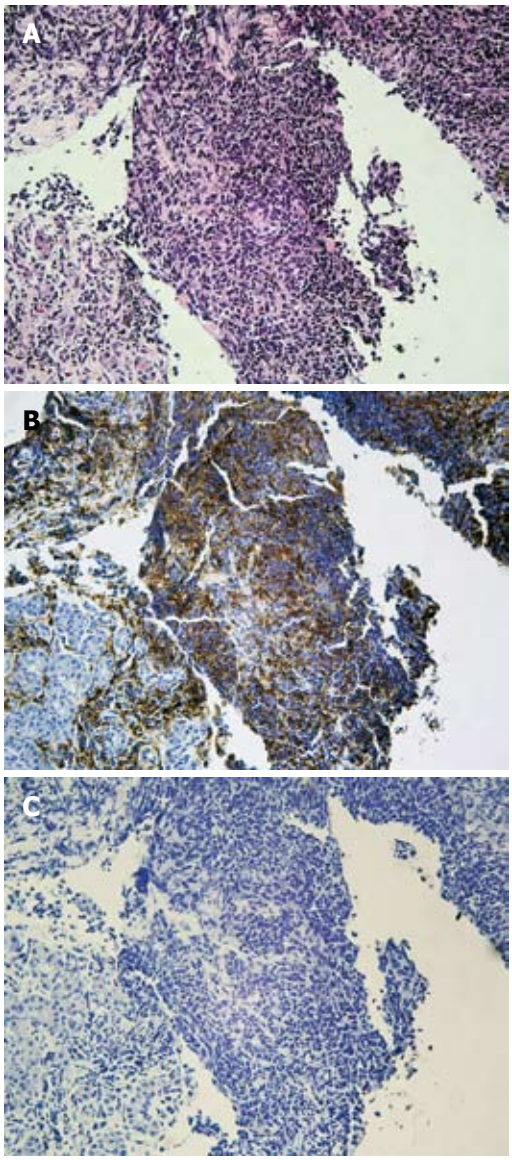

Figure 4 Histopathology of a specimen taken from the pancreatic head mass showed no malignant cells that indicated cancer. A: Marked infiltration of pancreatic parenchyma by lymphocytic mononuclear cells, as well as interstitial fibrosis suggested a diagnosis of AIP (HE, × 200). B: These infiltrated cells were revealed to be mainly T lymphocytes (T-cell staining, × 200). However, the histopathology was not diagnostic without so-called LPSP with scarce plasma cell infiltration. C: Immunohistochmistry using antibody against IgG4 resulted in a negative study (IgG4 staining, × 200).

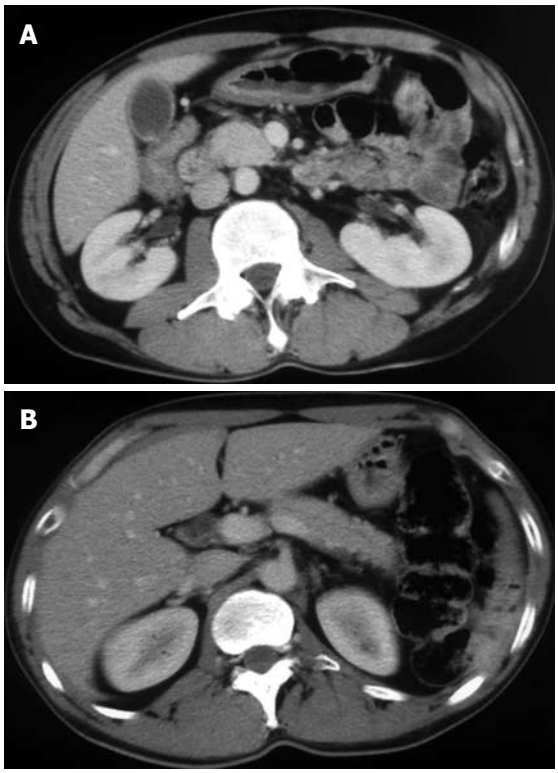

Figure 5 Abdominal enhanced CT scan with contrast medium in July 2007. As compared with CT imaging taken in May 2007, swelling of the pancreatic head had significantly regressed (A). Irregular dilatation of the main pancreatic duct had improved with the whole body bulk being reduced (B).

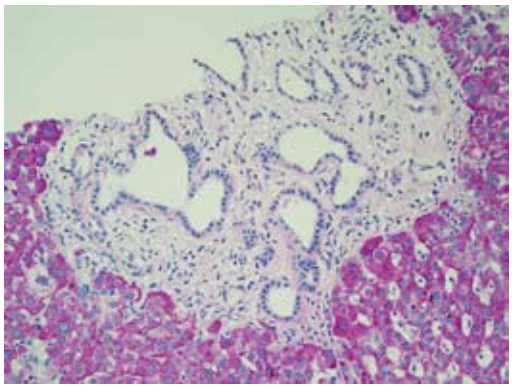

Figure 6 Histopathology of specimens taken from the hepatic parenchyma (PAS, × 200). Distribution of many irregular, angulated duct structures was observed. Ductal epithelium was comprised of monolayered columnar epithelium, morphologically identical to biliary epithelium, surrounded by fibrous connective tissues with minimal inflammatory change. The sequential histopathological features were considered to be bile duct hamartoma (VMC). Subsequent immunohistochemistry of the region by anti-IgG4 antibody was negative.

United States[11], and have been used clinically in each setting. However, there are some differences between each criterion for AIP that make it difficult to compare data in studies from different centers. Recently in 2008, Asian diagnostic criteria for AIP have been proposed by RCIPD and the Korean Society of Pancreatobiliary Diseases to reach an international consensus, thus enabling more cases to be accurately diagnosed and restraining the routine use of steroids for the diagnosis of AIP[21]. In the JPS criteria established in 2002[7], on the basis of the minimum consensus features of AIP required to avoid misdiagnosing malignancy, but not to pick-up suspicious cases of AIP, the demonstration of diffuse swelling of the pancreas and diffuse and irregular narrowing of the main pancreatic duct by ERCP is stressed and is required first of all. On revision of the JPS criteria in 2006[9], more cases of AIP have been correctly diagnosed. However, in the present case, since pancreatic swelling was located in only the head portion, which resembled a solid tumor, and there were neither pancreatic duct narrowing nor immunological abnormalities including IgG4, AIP could not be definitely diagnosed even with application of the revised Japanese criteria.

There are an increasing number of reports dealing

with atypical cases of AIP with focal swelling mimicking pancreatic cancer, as well as cases that begin with focal mass and progress to diffuse swelling, which results in a typical AIP appearance[16-19]. However, the most important issue when dealing with localized mass-forming AIP is that we should differentiate it absolutely from pancreatic or biliary cancer, in order to prevent too ready use of steroids, and leaving cancer untreated under a mistaken diagnosis. With this in mind, we performed US-guided percutaneous fine-needle aspiration biopsy, which targeted the pancreatic head mass and showed massive inflammatory cell infiltration, as well as interstitial fibrosis, and denied the presence of malignant cells. Subsequent steroid therapy resulted in an excellent clinical course of the patient. As AIP is known to show favorable prognosis, as in the present case, when steroid therapy is adequately administered[20], we should also detect suspicious variant forms of AIP before a patient undergoes invasive surgery.

It is debatable whether the diagnosis of AIP can be made from pancreatic core biopsy specimens, because they often do not show the complete spectrum of diagnostic change of lymphoplasmacytic sclerosing pancreatitis (LPSP) seen in typical AIP by routine histology alone[22]. In addition, certain types of pancreatic cancer exhibit a reaction that resembles LPSP[21]. However, we think that the purpose of fine-needle aspiration biopsy is to rule out the presence of malignant cells, and to provide a basis for the introduction of steroid therapy, rather than to establish a diagnosis of AIP, when we encounter a patient with a pancreatic mass, in whom differential diagnosis is difficult. Therefore, when a patient presents with uncertain clinical features of a pancreatic tumor, the presence of AIP and pancreatic fine-needle aspiration biopsy should be considered as one of the diagnostic modalities.

As awareness of AIP increases and more cases are reported and clinically investigated, high serum IgG4 concentration has been recognized as a notable characteristic and a marker of AIP disease activity[12]. Moreover, as the presence of a variety of extrapancreatic lesions related to IgG4-positive plasma cell infiltration, such as sclerosing cholangitis[23], sialadenitis[24] and retroperitoneal fibrosis[25], becomes evident, AIP has been proposed as one of the clinical aspects of generalized autoimmune IgG4-related sclerosing disease[13-15]. Therefore, we should consider the presence of extrapancreatic lesions of AIP when encountering unexplained manifestations outwith the pancreas. In the present case, the initial features of diffusely scattered intrahepatic lesions demonstrated on US and MRCP first led us to consider them to be possible extrapancreatic lesions of AIP. However, subsequent liver biopsy showed the presence of a unique group of cystic and dilated bile ducts surrounded by a dense fibrocollagenous stroma, which was revealed to be biliary hamartoma. Biliary hamartomas, also referred to as VMC, are benign liver malformations that histopathologically include a cystic dilated bile duct, typically 0.1-0.3 cm in diameter, embedded in abundant fibrous stroma[26]. As a result of its asymptomatic nature, this relatively rare entity is usually detected incidentally in 5.6% of reported autopsies[27]. In view of the results of histopathological investigation of the liver, including negative IgG4 staining, it seems unlikely that a common pathogenesis underlies AIP and VMC. However, as far as we know, this is the first presentation dealing with both AIP and VMC.

In conclusion, we reported herein a variant form of AIP accompanied by VMC, which did not fulfill the Japanese diagnostic criteria. When we encounter a patient with atypical presentation of a pancreatic disease and find it difficult to make a diagnosis, despite applying diagnostic criteria, fine-needle aspiration biopsy of the pancreatic lesion should be considered as a diagnostic modality.

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CASE REPORT

Multifocal intraductal papillary mucinous neoplasm of the pancreas-A case report

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Abstract

Cystic neoplasms of the pancreas are relatively rare, comprising 10 percent of pancreatic cysts and only 1 percent of pancreatic cancers. Cystic neoplasms include mucinous cystic neoplasms, serous cystadenomas, papillary cystic tumors, cystic islet cell tumors and intraductal papillary mucinous neoplasms of the pancreas (IPMNs). IPMN was first described in 1982. It has been most commonly described in 60 to 70 years old males, and represents a relatively "new" but increasingly recognized disease. The improvement and widespread use of modern imaging equipments and heightened awareness of physicians contribute to the increasing incidence of IPMN. The majority of IPMNs are located in the pancreatic head (75%) while the rest involves the body/tail regions. Multifocal IPMNs have been hypothesized, but the true presence of multifocality is unknown. Here we present a 72-year-old male diagnosed with IPMN (carcinoma *in situ*) in the pancreatic head and a branch duct type IPMN (duct atypia) in the pancreatic body and tail. The patient underwent a Whipple intervention and a distal pancreatectomy. A three-year disease-free survival has been observed so far.

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Key words: Intraductal papillary mucinous neoplasm; Pancreas; Multifocal; Pancreatectomy; Intraductal papillary mucinous neoplasms

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INTRODUCTION

Cystic neoplasms of the pancreas are relatively rare, comprising 10 percent of pancreatic cysts and only 1 percent of pancreatic cancers. Cystic neoplasms encompasses mucinous cystic neoplasms, serous cystadenomas, papillary cystic tumors, cystic islet cell tumors and intraductal papillary mucinous neoplasms of the pancreas (IPMNs)[1,2].

IPMN was first described in Japan in 1982, when four patients, diagnosed with pancreatic carcinoma with favorable outcomes, were reported sharing three distinct clinical features: dilated main pancreatic duct, patulous ampullary orifice and mucus secretion[3]. Since then, several reports have described mucus-producing pancreatic tumors with different stages of carcinogenesis, referred to by a variety of names[4-10], but mostly as mucinous ductal cancers. IPMN has very clear malignant potential and exhibits a broad histological spectrum, ranging from minimal mucinous hyperplasia to adenoma to invasive carcinoma[11-13].

The criteria used to classify IPMNs and to discriminate them from other mucin-producing cystic neoplasms of the pancreas have been established by the WHO in 1996[14]. The WHO gave a clear definition for IPMNs as intraductal mucin-producing neoplasms with tall, columnar, mucin-containing epithelium, with or without papillary projections. This type of neoplasm involves the main pancreatic duct and/or major side branches.

The majority of IPMNs are located in the pancreatic head (75%), while the rest involves the body/tail regions[15]. Multifocal neoplasms have been hypothesized[15], but the real prevalence of multifocality is unknown. Here, we present a 72-year-old male diagnosed with non-invasive

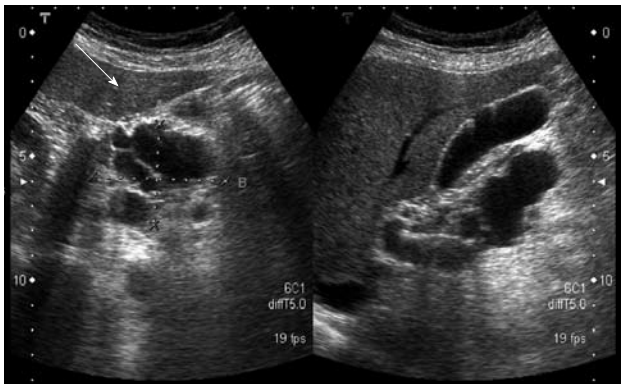

Figure 1 Abdominal ultrosonograpy showing multiple cystic tumors over the pancreatic head area with P-duct dilatation.

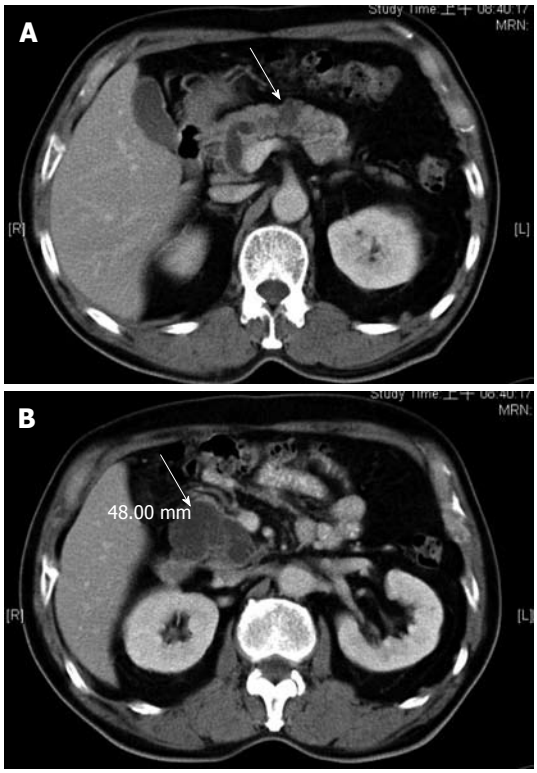

Figure 2 Abdominal computed tomography showing pancreatic head and body cystic tumors (arrow) with no evidence of regional lymphadenopathy or distant metastases.

mixed type IPMN (carcinoma *in situ*) in the pancreatic head together with a branch duct type IPMN (duct atypia) in the pancreatic body and tail.

CASE REPORT

A 72 years old male presented in 2005 with abdominal fullness after meals, general weakness, and body weight loss of about 2-3 kg occurred during the previous 3 mo. He had had diabetes and hypertension under regular medical control for the past 8 years. He was first admitted as outpatient, and an upper panendoscopy was performed, which showed chronic duodenal ulcer and gastric erosions. Antacids were prescribed but symptoms were not relieved.

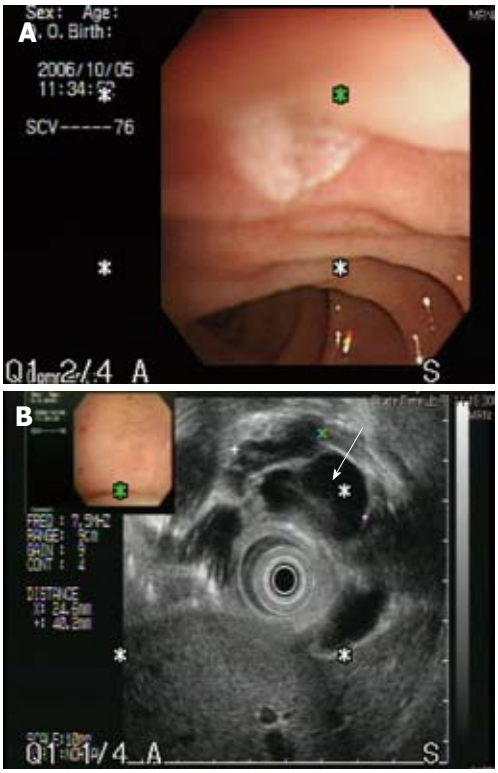

Figure 3 The endoscopic ultrasonogaphy showed that the papilla of Vater was grossly normal without mucin discharge. However, a cystic tumor (arrow) measuring 4.0 cm x 4.2 cm in size was seen over the pancreatic head. The lining of cysts showed no definite projectile mass and the pancreatic duct was dilated about 0.6 cm in the head and 0.4 cm in the body and tail area.

Abdominal ultrosonography was then carried out (Figure 1) and a pancreatic head cystic tumor with pancreatic duct dilatation (0.75 cm) was found. He was then hospitalized for further treatment. The physical examination revealed a soft abdomen without palpable mass. No lymphadenopathy was found over his neck or the axillary and inguinal areas. Digital examination showed nothing abnormal. The following laboratory tests were performed: white blood cell count 9000/mm^3, hemoglobin 13.5 g/dL, platelet count 257 000/mm^3, albumin 4.1 g/dL, aspartate aminotrasferase (AST) 16 U/L, alanine aminotransferase (ALT) 14 U/L, alkaline phosphatase 47 U/L, total bilirubin 0.6 mg/dL, creatinine 0.7 mg/dL, amylase 117 U/L, lipase 48 U/L. Tumor markers, including carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9), were also checked and their results were 0.92 ng/mL (normal < 5 ng/mL) and 27.2 U/mL (normal < 37 U/mL), respectively. Then abdominal computed tomography (CT) was performed (Figure 2), which showed two cystic tumors at the level of the pancreatic head and body, measuring 4.8 cm and 1.2 cm, respectively. To further investigate the nature of the tumors, endoscope ultrosonography (EUS) and magnetic resonance imaging (MRI) were carried out. The EUS (Figure 3) disclosed a cystic tumor, 4 cm × 4.2 cm, without obvious internal projective mass in the pancreatic head. The papilla of Vater was grossly normal without mucin discharge. The MRI (Figure 4) showed three cystic lesions in pancreatic head (4.9 cm), body (1.8 cm) and

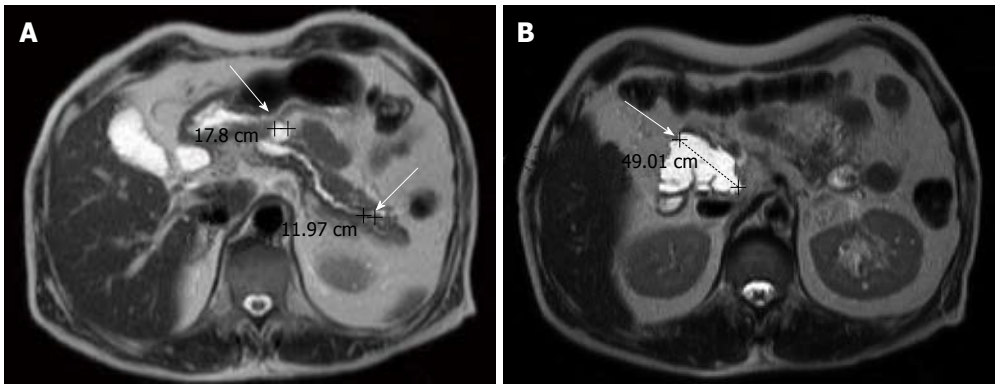

Figure 4 Magnetic resonance imaging showing three cystic lesions in the pancreatic head (4.9 cm), body (1.8 cm) and tail (1.2 cm), with P-duct dilatation. Mild common bile duct and common hepatic duct dilatation without definite tumor was observed in the liver upon MRI.

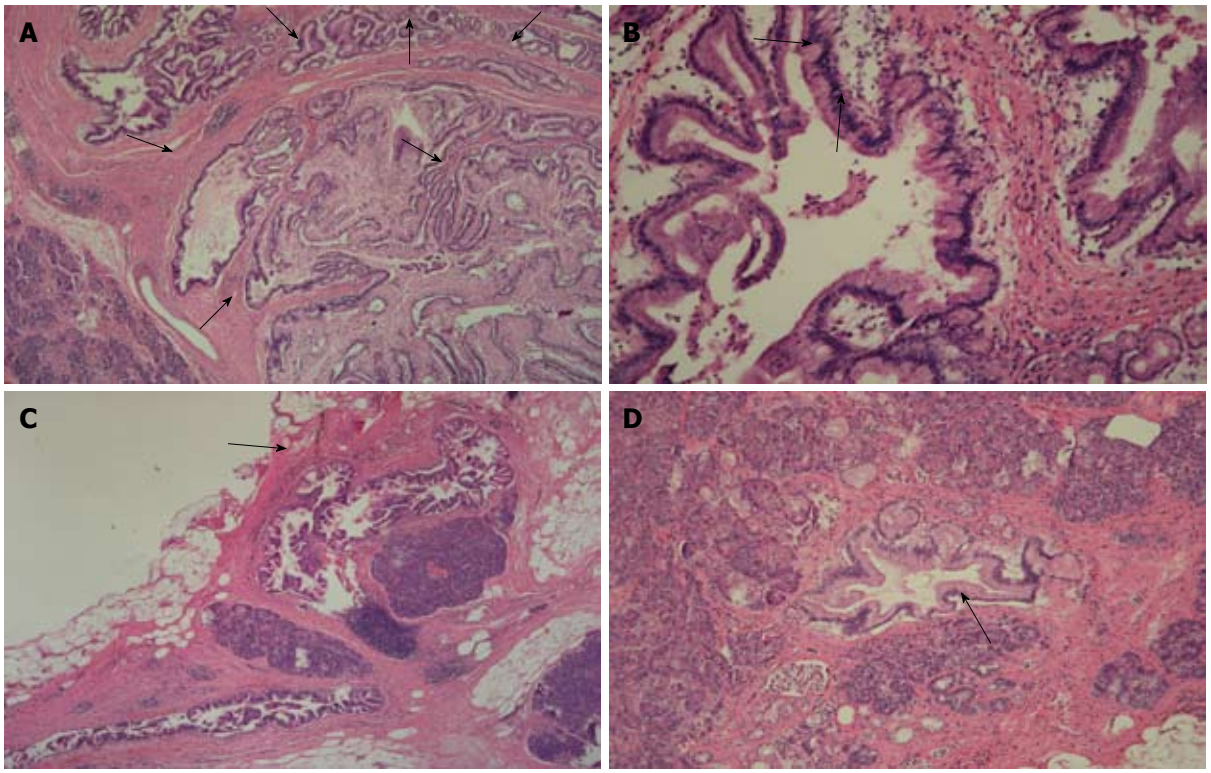

Figure 5 Histopathologic findings (HE × 200). A: Histopathological examination showed the presence of cystically dilated ducts with formation of papillary growth lined by mucin-secreting columnar epithelium at the level of the pancreatic head; B: The mucin-secreting epithelium reveals stratification of atypical nuclei. No obvious stromal invasion was seen and the features suggested a diagnosis of intraductal papillary mucinous carcinoma, noninvasive type; C: The section of the pancreatic body showed that it was focally involved by the lesion (arrow); D: The section of the pancreatic tail showed the focal presence of atypical ducts.

tail (1.2 cm) with pancreatic duct dilatation. Given the presence of pancreas cystic neoplasms, which may be malignant IPMNs, cystic adenocarcinomas or cystic adenomas, affecting the pancreatic head, body and tail, an extended Whipple operation (including the partial resection of the pancreatic body, about 2 cm away from the portal vein) together with a distal pancreatectomy were performed. Intraoperative findings revealed three tumors as described in the MRI. Intraoperative pancreatic US revealed no additional cystic mass in the residual pancreas, measuring about 7 cm in length, after resection. The proximal and distal margins were all negative for malignancy demonstrated by intraoperative histopathologic examination. The regional lymph nodes were grossly normal.

Histopathologic examination revealed that the pancreatic head showed the presence of cystically dilated ducts, encompassing the main and branch ducts, with formation of papillary growth lined by mucin-secreting columnar epithelium. The mucin-secreting epithelium had stratification of atypical nuclei. No obvious stromal invasion was seen. The features suggested a diagnosis of intraductal papillary mucinous carcinoma, non-invasive type (Figure 5A, B). The pancreatic body was focally involved by the lesion (Figure 5C), in the branch duct, not contiguous to the lesion of the pancreatic head. The pancreatic tail displayed focal presence of atypical ducts (Figure 5D), located in the branch duct. The final pathological diagnosis was non invasive mixed (involving both the main and the branch duct) type IPMN (carcinoma *in situ*) in the pancreatic head and branch duct type IPMN (duct atypia) in the body and tail. The postoperative course was uneventful and a three-year disease free survival has been achieved so far.

DISCUSSION

An enormous increase in the diagnosis of IPMN has occurred in recent years[16-18], which is due both to the improvement and widespread use of modern imaging equipments and to a heightened awareness among physicians.

IPMN usually occurs in 60 to 70 years old males, most of whom have a long-standing history of recurrent acute pancreatitis or symptoms suggestive of chronic obstructive pancreatitis, similarly to our patient, due to intermittent obstruction of the pancreatic duct with mucus plugs[19]. Other symptoms are nonspecific[20,21] and no signs or symptoms are pathognomonic of primary cystic neoplasms of the pancreas, including IPMN. Furthermore, many patients with no symptoms have their tumors detected incidentally at imaging studies performed for unrelated indications. Thus, there is often a several months delay in the diagnosis of IPMN, due to the insidious nature of this disease and to the lack of awareness of this entity among physicians[13,20,22-24]. Some patients may have elevations of pancreatic enzymes with episodes of pain (with or without biliary obstruction), but in most cases routine laboratory tests are normal. Similarly, tumor markers, such as CA 19-9 and CEA, are elevated in less than one-fifth of cases only, and are not always indicative of a malignant transformation of the tumors[19]. In this case, there were normal routine laboratory tests and tumor markers.

IPMN is a precancerous lesion with a well described adenoma-carcinoma sequence. However, the rate of progression appears to be extremely slow (approximately 15 to 20 years)[25].

The IPMN has been classified as main duct type (MDT-IPMN) or branch duct type (BDT-IPMN), based upon the anatomic involvement of the pancreatic duct[26,27]. The majority of IPMNs are located in the pancreatic head (75%) whereas the rest involves the body/tail regions[15]. Multifocal neoplasms have been hypothesized[15], but the real prevalence of multifocality is still unknown. The final diagnosis of this case proved the existence of this rare entity.

The majority of patients with IPMN does not have invasive cancer, and usually have a prolonged course without the development of cancer. In one series, the ten-year actuarial risk of high grade dysplasia and invasive cancer were 49% and 29%, respectively[28]. The five-year actuarial risk of progression was significantly higher in patients with main duct type compared with those with exclusively the branch duct type (63% *versus* 15%). Surgery is the only treatment option in patients who do have high grade dysplasia or cancer detected by endoscopic or cytological examination. Preoperative staging, using a combination of ERCP, pancreatoscopy, EUS and intraductal ultrasonography, may be beneficial to determine the indication to surgery[29-32]. However, accurate prediction of malignancy is not possible preoperatively. Sugiyama *et al*[33] proposed some predictive factors of malignant IPMN, including (1) tumor size, since an IPMN > 4 cm and a branch duct IPMN > 3 cm suggest malignancy; (2) a mural nodule of a size > 5 mm; (3) a dilatation of main pancreatic duct > 10 mm; (4) the patient age, the mean age for adenomas being 64, whereas that of invasive cancers is 67; and (5) symptoms, especially jaundice.

Selection of a surgical procedure for treating IPMN remains controversial, because IPMNs show a wide spectrum of histologic changes, ranging from hyperplasia to invasive carcinoma and because of the so far unresolved question of multifocality. As demonstrated in this patient, the aim of surgery is to achieve complete resection with negative margins for invasive and noninvasive disease and to minimize the incidence of recurrence in the pancreatic remnant. However, an extended resection of the pancreas may pose the risk of inducing a severe pancreatic functional insufficiency, both exocrine and endocrine.

Another difficult question is the management of patients with suspected IPMNs who do not have high grade dysplasia or cancer detected during initial evaluation, and of patients with small (< 3 cm) branch-duct IPMNs. Our opinion is that management should be based upon the confidence in the diagnosis, the presence of symptoms and a discussion with the patient regarding the possible risk of subsequent malignant transformation. Surgery can be reasonably offered to patients who are good surgical candidates in order to confirm the diagnosis and to prevent the subsequent development of cancer. In contrast, patients who are poor surgical candidates can be treated conservatively with an imaging test performed every six to twelve months to evaluate the stability of the lesion. Our case fared very well despite the fact that he was 72 years old. Originally, we suggested the resection of pancreatic head with close follow-up of the other two tumors, considering the tiny risk of malignant transformation, but both the patient and his family preferred a complete resection of the three tumors. Finally, we performed an extended Whipple operation (including the partial resection of the pancreatic body) with a distal pancreatectomy. The postoperative course was uneventful without deterioration of the diabetes and exocrine insufficiency.

The final pathologic diagnosis was noninvasive mixed (involving both the main and the branch duct) type IPMN (carcinoma *in situ*) affecting the pancreatic head and a branch duct type IPMN (duct atypia) of the pancreatic body and tail. This confirmed the occurrence of multifocality of IPMNs of the pancreas. The patient has until now achieved a three year disease free survival.

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CASE REPORT

Pulmonary and cerebral lipiodol embolism after transcatheter arterial hemoembolization in hepatocellular carcinoma

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Abstract

Pulmonary and cerebral lipiodol embolism after transcatheter arterial chemoembolization (TACE) of hepatocellular carcinoma is rare. To our knowledge, only 7 cases have been reported in the literature. We present a case of pulmonary and cerebral lipiodol embolism, and analyzed retrospectively the imaging and clinical data of the patient and conclude the most probable mechanism of pulmonary and cerebral lipiodol embolism, which is different from that of the cases reported previously.

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Key words: Pulmonary embolism; Cerebral embolism; Lipiodol; Transcatheter arterial chemoembolization; Hepatocellular carcinoma; Complication

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INTRODUCTION

Transcatheter arterial chemoembolization (TACE) has been widely used in the treatment of primary hepatocellular carcinoma (HCC). The reported rare or serious complications associated with TACE mainly include: acute hepatic failure, liver abscess, hepatic infarction, hepatic artery occlusion, spontaneous rupture of tumor, gallbladder infarction, perforation of duodenum, acute renal failure, gastrointestinal mucosal ulceration, *etc*[1,2]. Cerebral lipiodol embolism is a rare complication of TACE. To our knowledge, only 7 cases of cerebral lipiodol embolism after TACE have been reported so far[3-7]. We herein report a case of pulmonary and cerbral lipiodol embolism after TACE in HCC, and discussed the possible mechanism, which is different from the cases reported previously.

CASE REPORT

A 36-year-old man who complained of right upper abdominal pain for one week was admitted to our hospital. He had a history of hepatitis B for 10 years. Hepatic computed tomography (CT) revealed a massive HCC in the right liver and tumor thrombosis in the right branch of portal vein. The concentration of blood α-fetoprotein (AFP) was 2.5 ng/mL, while hepatic function was classified as A according to Child-Pugh classification. The surgical resection cannot be performed because of inadequate residual liver volume. So the patient underwent the first TACE.

As revealed by angiography, a huge hypervascular tumor located in the right liver was only supplied by the right hepatic artery (RHA) without arteriovenous shunt. TACE was performed *via* the RHA using a mixture of 40 mg pirarubicin, 40 mL lipiodol, 20 mg hydroxycamptothecine and 150 mg Oxaliplatin. Gelatin sponge particles (560-710 μm) were used to reduce the flow of tumor feeding artery. After the operation, the patient had a sustained high fever in the afternoon with a maximum of 39.6℃ for 10 d. A follow-up non-contrast hepatic CT showed incomplete lipiodol retained in the hepatic tumor and hyper-attenuating lipiodol deposition only in the right collapsed basal lung (Figure 1). The patient recovered and was discharged from hospital two weeks after TACE. Four weeks later, a follow-up hepatic CT demonstrated that incomplete lipiodol deposited in

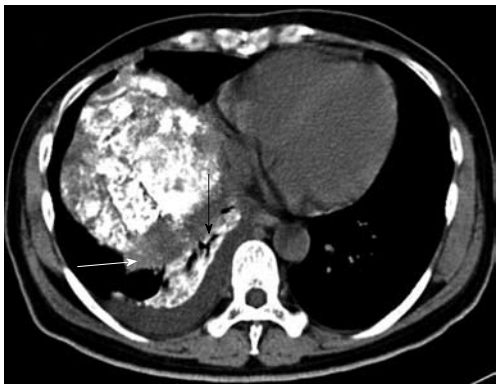

Figure 1 Ten days after the first TACE, a non-contrast hepatic CT revealed incomplete lipiodol retained in hepatic tumor (white arrow) and hyper-attenuating lipiodol deposition only in the right collapsed basal lung (black arrow).

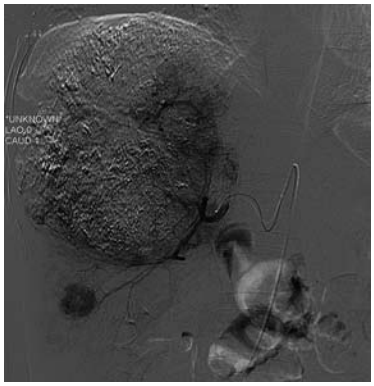

Figure 2 During the second TACE, hepatic angiography revealed the residual tumor fed by RHA and no arteriovenous shunt was found.

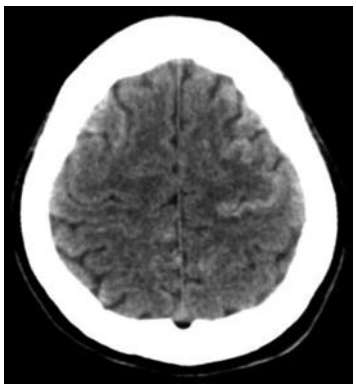

Figure 3 Fifty-three hours after the second TACE, a non-contrast head CT showed multiple high-attenuating spots and patch-like lesions in bilateral parietal and frontal cortex, suggesting the lipiodol desposition.

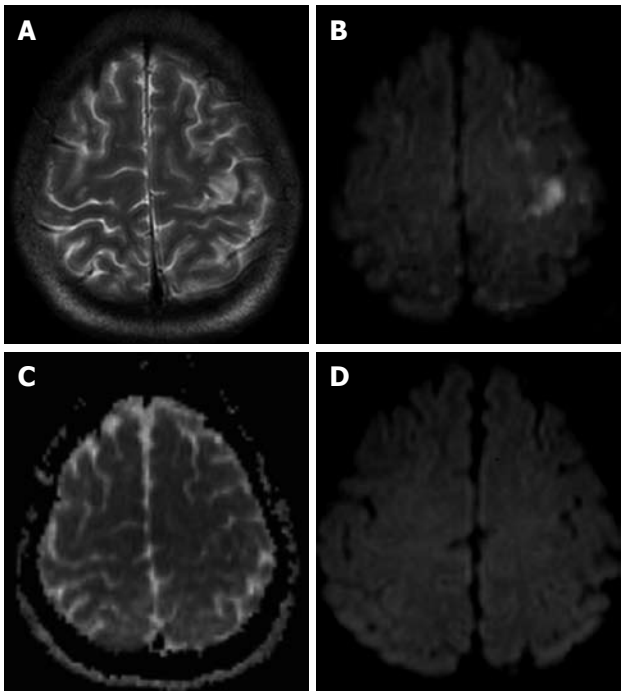

Figure 4 A 36-year-old man with cerebral lipiodol embolism. A: Axial T2-weighted imaging (T2WI); B: Biffusion-weighted imaging (DWI); C: Apparent diffusion coefficient (ADC); D: Follow-up DWI. A, B and C obtained 57 h after the second TACE, and D obtained 5 wk after the second TACE. T2WI and DWI (A, B) demonstrated multiple high-signal lesions on the bilateral frontal and parietal lobe. The corresponding lesions showed a low signal area on the ADC map (C). A follow-up DWI revealed no obvious abnormal signal (D).

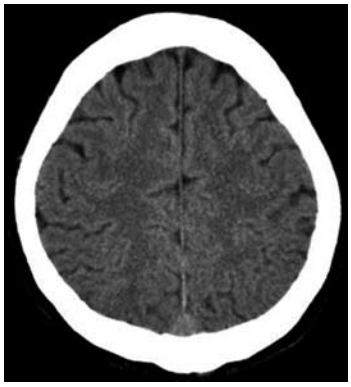

Figure 5 A follow-up head CT obtained 5 wk after the second TACE showed no abnormality and the previously observed lipiodol deposition cleared entirely.

the tumor, so the second TACE was performed. During the second procedure, the hepatic artery angiography revealed that the residual tumor was supplied by RHA, but no arteriovenous shunt was found (Figure 2). A mixture of 40 mg pirarubicin, 40 mL lipiodol, 20 mg hydroxycamptothecine and 150 mg Oxaliplatin was infused the RHA. The embolism process was monitored with fluoroscope and no abnormal flow of the lipiodol was found. Postoperative abdominal X-ray plain film showed dense lipiodol deposition in the tumor. The patient felt transitory numbness in the right hand at the end of the procedure. Two hours after the 2nd TACE, the patient had a numbness and weakness in the right hand and the right fingers could not carry out fine motor activities (such as writing, holding chopsticks, finger' s diadochokinesia, *etc.*), no symptom of chest tightness and dyspnea occurred. A non-contrast head CT obtained in 53 h after the second TACE showed multiple high-attenuating spots and patch-like lesions in bilateral parietal and frontal cortex (Figure 3). At the same time, a non-contrasted thoracic CT demonstrated small doses of hyper-attenuating lipiodol deposition in the bilateral basal lungs. Cerebral magnetic resonance imaging (MRI) was performed 57 h after TACE by 1.5 T magnetic resonance imaging (Siemens), diffusion-weighted imaging (DWI) and T2WI demonstrated multiple high-signal lesions on the bilateral frontal and parietal lobe (Figure 4A and B); the corresponding lesions showed a low signal area on the apparent diffusion coefficient (ADC) map (Figure 4C). Pulmonary and cerebral lipiodol embolism with acute cerebral infarction after TACE was considered. After the intervention of positive dehydration, diuresis and nutrition of nerve for 10 d, the right hand resumed normal function. Four weeks later, a follow-up head CT showed no abnormality and the previously observed

lipiodol deposition had been cleared entirely (Figure 5). Follow-up MRI revealed no obvious abnormal signal on the DWI (Figure 4D). After the patient underwent TACE, the volume of the normal left liver had a marked compensatory hyperplasia, which was sufficient for safe resection. Surgical tumor resection was performed. During the procedure, the right hepatic massive tumors were seen invading into the diaphragm. Pathological diagnosis revealed primary HCC with large necrosis, surrounded by nodule hepatic cirrhosis. The patient is still living in good health.

DISCUSSION

Cerebral lipiodol embolism rarely occurs after TACE in HCC. There are only seven reported cases and its possible mechanism was analyzed in detail in three cases. Wu *et al*[5] thought the link between systemic vessels and pulmonary vessels may occur *via* the adhesive pleurae or tumor invasion into the thoracic cavity. The lipiodol injected *via* the inferior phrenic artery may enter the pulmonary circulation, and then entered the systemic circulation *via* these pathways, resulting in cerebral lipiodol embolism. Matsumoto *et al*[6] concluded that communication between tumor feeding artery and pulmonary vein might occur *via* adhesive pleural or tumor invasion into the diaphragm. Therefore, a small dose of lipiodol could enter the systemic circulation quickly and caused cerebral embolism. Choi *et al*[7] concluded that a large dose of lipiodol and communication between right inferior phrenic artery and pulmonary vessels were contributed to cerebral lipiodol embolism. In this report, we analyzed retrospectively the features of the case as follows. (1) The tumor is huge and was adhered to the diaphragm, suggesting the tumor invading into the diaphragm, which was confirmed during the surgery. (2) The repeated hepatic angiography did not reveal any arteriovenous shunt. (3) The pulmonary lipiodol embolism was found only in the right collapsed basal lung and no neurological signs or symptom of cerebral lipiodol embolism appeared after the first TACE, which suggested that the communication between the tumor feeding artery and the branch of the right pulmonary artery might occur and congenital intrapulmonary arteriovenous shunt did not exist in the field at that time. Given the communication between the tumor feeding artery and pulmonary vein, cerebral lipiodol embolism may occur directly and pulmonary lipiodol embolism in both lung fields. Due to the communication between the tumor feeding artery and hepatic vein, pulmonary lipiodol embolism might be found in both lung fields. So these two possibilities were excluded. (4) The symptom of cerebral lipiodol embolism appeared immediately after the second TACE. According to the features mentioned above, we conclude the most probable mechanism of pulmonary and cerebral lipiodol embolism as follows: Since the tumor invaded into the diaphragm, the communication between the tumor feeding artery and the branches of the right pulmonary artery were presented, and the lipiodol went into the branches of the right pulmonary artery through the communication, resulting in pulmonary lipiodol embolism during the first TACE. While there was no congenital intrapulmonary arteriovenous shunt, the cerebral lipiodol embolism did not form after the first TACE. However, the second intrapulmonary arteriovenous shunt appeared during the pulmonary lipiodol embolism because of increasing pulmonary artery pressure or hypoxia. Therefore, the cerebral lipiodol embolism occurred immediately after the second TACE.

In this case, the head CT revealed multiple hyperattenuation spots and patch-like lesions in the bilateral frontal and parietal cortex, which suggested hyperattenuating lipiodol deposition. Cerebral MRI demonstrated multiple ischemic lesions in the bilateral frontal and parietal lobe but not lipiodol deposition. In CT and MRI embolism is observed mainly in the gray matter and the pathological changes are predominant in the white matter[8]. Therefore, CT is considered useful to the diagnosis of cerebral lipiodol embolism, and DWI can help detect lesions and evaluate the therapeutic effect.

Pulmonary and cerebral lipiodol embolism is a rare and serious complication. In this case, pulmonary lipiodol embolism only occurred in the right lung, suggesting the communication between tumor feeding artery and branch of the right pulmonary artery. The risk of pulmonary and cerebral lipiodol embolism should be considered in the tumor treated with TACE again. To prevent the pulmonary and cerebral lipiodol embolism, it is important to consider an individualized therapeutic plan, including the dose of lipiodol and evaluation for the presence of shunt prior to TACE.

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Meetings

Events Calendar 2009

January 12-15, 2009
Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

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

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

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

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

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

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

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

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

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

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

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

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

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

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

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

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

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

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

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

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

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

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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Issue with no volume

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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

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

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Electronic journal (list all authors)

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

Patent (list all authors)

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

Statistical data

Write 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 υ (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

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
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EDITORIAL

A shield against a monster: Hepatitis C in hemodialysis patients

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Abstract

Hepatitis C virus (HCV) infection is highly prevalent among patients on hemodialysis (HD). The prevalence of HCV infection in HD patients varies markedly from country to country. Some factors are especially related to these high prevalence rates, such as blood transfusions and length of dialysis time. Nosocomial routes of transmission including the use of contaminated equipment and patient-to-patient exposure is considered more important. Several prophylactic measures have been suggested to avoid infection by HCV in the HD environment.

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INTRODUCTION

Hepatitis C virus (HCV) infection is considered a major public health problem worldwide. Patients with chronic renal failure who are on hemodialysis (HD) have a high prevalence of HCV. They are among the highest risk groups for the acquisition of HCV infection[1-3]. Prevalence of HCV infection has decreased in this group in recent years[4], but still remains a significant public health concern. HCV-infected patients on HD have significant liver disease and a decreased life expectancy[5,6]. The relative risk for death in HCV-infected patients on HD compared with non-infected patients is greater than 1.4[6-8]. In addition, HCV infection leads to decreased graft and patient survival in renal transplant recipients[9,10]. On the other hand, due to the increased prevalence of non-communicable diseases such as diabetes mellitus and hypertension, and their complications, chronic renal failure has become a more serious health issue throughout the world[11]. Therefore, the clinical importance of HCV has been increasingly recognized in the dialysis community.

PREVALENCE AND INCIDENCE

HCV prevalence in HD varies geographically, both within and between countries[12]. The reported anti-HCV seropositivity since 1999 ranges from low (1.9%) in the Slovenian 2001 Annual Report[13] to high (80%) in Senegal[14]. HCV seroprevalence in the HD population was 59% in Bosnia and Herzegovina[15] , 6.8% in Belgium[16], 16.3% in France[2], 6.1% in Germany[17], 10%-29% in Greece[18-20], 22.5%-32.1% in Italy[21,22], 75% in Moldavia[23], 3.4% in the Netherlands[24], 1.9% in Slovenia[13], 11% in Sweden[25], 7%-23.3% in the USA[26-30], 4% in the UK[31] 20.5% in Libya[32], 71% in Kuwait[31] 80% in Senegal[14], 23.7% in Sudan[33], 19%-41.7% in Tunisia[34,35], 8.4%-43.2% in Brazil[36-39], 6.7% in Mexico[40], 59.3% in Peru[41], 3.5% in Puerto Rico[42] and 13.2% in Iran[43]. Some investigators have suggested a decline in HCV prevalence among HD patients in recent years, mostly attributable to strict adherence to universal precautions with[16,44-49] or without[50,51] observing isolation measures. This decrease is more significant in the USA and European countries[4,16,47,52]. Studies that have prospectively followed HD patients for their HCV serostatus have yielded annual incidence rates of de novo HCV infection of 0.4% in France[53], 0.5% in Tunisia[54], 0.5% in the Netherlands[24], 0.83% in Italy[55], 1.38%[56] and 2.1%[57] in the USA, 0.33%[58], 2.59%[59], 3.1% in Japan[60], 3.7%[61] 5.5% in Brazil[62], and 6.2% in Greece[20]. This variation in different countries and different centers underlines the importance of infection

control. New infection was evidently more frequent at centers that had higher anti-HCV prevalence and failure in infection control measures. In some countries, both prevalence and incidence remain very high, indicating major ongoing nosocomial transmission, probably due to the limited resources available to treat a rapidly growing HD population[63,64].

RISK FACTORS FOR HCV TRANSMISSION

The high prevalence of HCV seropositivity among HD patients was initially attributed to blood transfusions for the treatment of uremic anemia in this population, which were often necessary[41,65-68]. Historically, the number of blood transfusions received was consistently reported in the literature to be associated with an increased prevalence of HCV-positive dialysis patients[31]. However, several recent reports have failed to recognize blood transfusion as an independent risk factor in HCV spread among HD subjects[2,20,23,24,30,33,62,69-74]. Indeed, from the late 1980s onward, the prescription of erythropoietin reduced the need for blood transfusion in HD patients. Furthermore, the introduction of sensitive tests for screening blood donors has markedly reduced the risk of HCV transmission through blood product transfusion. These two reasons may explain recent findings on the lack of association between blood transfusion and HCV infection. Nonetheless, considering the fact that new HCV infections do still occur in patients without a history of transfusion, the duration of HD is increasingly being considered as a risk factor for HCV infection[75,76]. Almost all recent surveys on the subject have congruently suggested that the length of time on HD is a risk factor for HCV seropositivity[17,20,23,29,30,33,36,39,43,60,69-71,77-80]. Nosocomial patient-to-patient transmission of HCV infection among HD patients has been suggested by several investigators who performed phylogenetic analysis of HCV viral isolates[24,25,53,54,81-84]. Lack of strict adherence to universal precautions by staff and sharing of articles such as multidose drugs might be the main mode of nosocomial HCV spread among HD patients[54,82,84-86]. Although some studies found that nosocomial spread of HCV declined when HCV-infected patients were treated in dedicated HD units[44-49,87,88], other investigators could control nosocomial spread of HCV among HD patients by the strict application of hygienic precautions, without isolation of HCV-infected subjects or machine segregation[12,50,89]. Indeed, the observed efficacy of isolation might simply be due to the prevented sharing of articles between patients and might reflect a better implementation of other hygienic precautions.

The spread of HCV infection in HD units is mainly due to nosocomial transmission between patients[53,88,90-94]. The importance of this route of transmission is demonstrated by the high HCV prevalence in some HD units and by the lower infection rate in patients on peritoneal dialysis compared with those on HD. A high prevalence of patients with HCV infection in HD facilities has been considered a risk factor for transmission of the infection. However, there is no consensus on the necessity for infection control isolation of HCV-positive patients for at least two reasons: firstly, the infectivity of HCV is lower than that of the hepatitis B virus; and secondly, the criteria for patients to be isolated remain to be defined. On the contrary, some HD patients are infected with HCV but do not have antibodies. Detection of viral RNA by reverse-transcription polymerase chain reaction (RT-PCR) is the only method to confirm HCV infection, however, this technique is not available at all centers.

PREVENTIVE STRATEGIES

Several prophylactic measures have been suggested to avoid infection by HCV in the HD environment, and range from isolating patients with HCV infection[44,47,48,88,95], to adopting a series of biosafety measures specific for HD, such as preparing medications in a separate area, cleaning and disinfecting dialysis station surfaces, washing hands and changing gloves between patient contacts, and items dedicated for use only with a single patient[12,39,50,96]. Strict adherence to universal infection control precautions seems to be enough to control the spread of disease in HD units[12,50,89,97-99]. Some reports have recommended the adoption of infection control isolation measures at centers with a high HCV prevalence[47,87,100,101] or if the staff/patient ratio at the center is lower than 28/100[102]. At centers with a high prevalence of HCV infection and in developing countries, universal precautions may not always be possible to implement. Thus isolation measures for HCV-positive patients should be implemented[47]. The CDC recommends that special precautions be observed in dialysis units including the wearing and changing of gloves and water-proof gowns between patients; systematic decontamination of the equipment, circuits, and surfaces after each patient treatment; no sharing of instruments (e.g. tourniquets) or medications (e.g. multiuse vials of heparin) among patients; and the assignment of patients to specific HD units[97]. Clearly, it is necessary to attempt, one step at a time, to minimize intradialytic or intracenter HCV transmission.

CONCLUSION

In summary, despite the marked decrease in anti-HCV prevalence in HD patients in many countries, the disappearance of HCV from HD units should not be expected for decades. Universal infection control precautions are the keystone in the prevention of nosocomial HCV transmission in HD units; however, isolation measures, including health care monitoring of infected patients and providing care in a dedicated section of the unit, improve prevention results. Those HD units with a high HCV prevalence or in which there is no fulltime infection control personnel dedicated to the infected patients during HD sessions may have a greater risk of seroconversion. Therefore, isolation in

special units or dialyzing patients in specific sessions must be considered[44]. As HCV-infected HD patients serve as a reservoir of infection for other patients, HD staff, and the transplant team[28,103,104] at HD centers must be aware of new HCV infections in order to review their practices and increase vigilance. Public health authorities should be aware about the prevalence and incidence of HCV infection in HD patients in different cities in their respective countries, so that changes can be proposed and the risks of infection among patients can be assessed. Implementation of surveillance systems and continuing education of the unit's personnel on recommended infection control measures in HD units are necessary. The treatment of most HCV-infected patients with interferon alpha can significantly contribute to decreasing HCV infection in this group in the future[105]. Successful control of infection requires further studies to assess the effectiveness of different preventive policies.

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TOPIC HIGHLIGHT

Salvatore Gruttadauria, MD, Associate Professor, *Series Editor*

Editorial statement

Salvatore Gruttadauria, Bruno G Gridelli

In the following four articles, we will provide an overview of the current clinical work in different areas of liver transplantation.

For many decades, this transplantation has been the treatment choice for patients suffering from chronic and acute liver diseases.

Understanding of the complexity of this procedure can be read only through a multidisciplinary approach.

Liver transplantation has become a clinical reality thanks to the pioneer Thomas E Starzl, MD, PhD, who at the University of Colorado was one of the first to test cyclosporine in humans. Considered the father of liver transplantation, he performed the world's first liver transplant at the University of Colorado in 1963. Upon his arrival in Pittsburgh in 1981, when the university's liver transplant program began, Dr. Starzl continued research on the drug, which was approved by U.S. Food and Drug Administration (FDA) in November 1983.

Now, after many thousands of liver transplants have been successfully accomplished worldwide, the main problems to be solved remain the chronic shortage of organs and the need to investigate alternative and less aggressive forms of therapy, for the cure of end-stage liver disease.

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TOPIC HIGHLIGHT

Salvatore Gruttadauria, MD, Associate Professor, *Series Editor*

Pediatric liver transplantation

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Abstract

In previous decades, pediatric liver transplantation has become a state-of-the-art operation with excellent success and limited mortality. Graft and patient survival have continued to improve as a result of improvements in medical, surgical and anesthetic management, organ availability, immunosuppression, and identification and treatment of postoperative complications. The utilization of split-liver grafts and living-related donors has provided more organs for pediatric patients. Newer immunosuppression regimens, including induction therapy, have had a significant impact on graft and patient survival. Future developments of pediatric liver transplantation will deal with long-term follow-up, with prevention of immunosuppression-related complications and promotion of as normal growth as possible. This review describes the state-of-the-art in pediatric liver transplantation.

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Key words: Pediatric liver transplantation; Indications; Surgical techniques; Complications

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INTRODUCTION

Liver transplantation has been very successful in treating children with end-stage liver disease, and offers the opportunity for a long healthy life. Organ scarcity, which is the main limitation to the full exploitation of transplantation, is being overcome thanks to innovative surgical techniques, and all children in need, even the youngest, today have the chance of being transplanted, with almost no waiting list mortality. Split-liver and living-donor transplantation have contributed to reversing a situation in which, during the 1980s and 90s, children had greater waiting list mortality compared to that of adult patients.

Several years ago, the main focus of care of children with end-stage liver disease was to find a liver transplant, but today, the main interest is in long-term follow-up, with prevention of immunosuppression-related complications and promotion of as normal growth as possible. The history of pediatric liver transplantation has clearly shown that success is dependent on strict and integrated collaboration between referring pediatricians, pediatric transplant hepatologists, transplant surgeons, nurses, transplant coordinators, psychologists and social workers. Everybody involved has the task of bringing a cure to a population of pediatric patients who present some of the most challenging clinical problems in modern medicine.

INDICATIONS FOR LIVER TRANSPLANTATION

The main indications for liver transplantation in the pediatric population are as follows: (1) Extra-hepatic cholestasis: biliary atresia. (2) Intra-hepatic

cholestasis: sclerosing cholangitis; Alagille's syndrome; non-syndromic paucity of intrahepatic bile ducts; and progressive familial intrahepatic cholestasis. (3) Metabolic diseases: Wilson's disease; α_1-antitrypsin deficiency; Crigler-Najjar syndrome; inborn error of bile acid metabolism; tyrosinemia; disorders of the urea cycle; organic acidemia; acid lipase defect; oxaluria type Ⅰ; and disorders of carbohydrate metabolism. (4) Acute liver failure. (5) Others: primary liver tumor and cystic fibrosis.

Cholestatic liver diseases

Typically, the child referred to a liver transplant center is a small baby with cholestatic liver disease. Out of 1187 children transplanted in North America between 1995 and May 2002, 33.5% were ⩽ 12 mo old at the time of transplantation, 55.6% had cholestatic disease, and 41.6% had biliary atresia. Of the children transplanted at < 1 year of age, 65.6% had biliary atresia[1]. Most of these children have undergone a Kasai procedure that failed to re-establish effective biliary flow, which caused rapid evolution to secondary biliary cirrhosis. When intrahepatic cholestatic diseases (Alagille's syndrome, progressive familial intrahepatic cholestasis, and sclerosing cholangitis) or sclerosing cholangitis are diagnosed, liver transplantation is indicated to eliminate severely debilitating symptoms, such as pruritus. Children affected by these diseases are also at high risk for the development of liver cancer[2].

Metabolic diseases

Metabolic diseases are the second most common indication for liver transplantation[3]. Metabolic diseases can be divided in two groups on the basis of the presence or absence of structural damage of the liver. To the first group belong α_1-antitripsin deficiency, tyrosinemia and Wilson's disease, which have the potential to progress to end-stage liver failure, liver cancer (Figure 1) and acute liver failure, while diseases such as Crigler-Najjar syndrome type Ⅰ and ornithine transcarbamylase (OTC) deficiency belong to the second group. In primary hyperoxaluria type Ⅰ, liver and kidney transplantation is considered when irreversible kidney damage from oxalic acid accumulation has developed. Different transplantation timings have been tested, combined liver and kidney transplantation (simultaneous or sequential) and pre-emptive liver transplantation (before end-stage renal failure occurs)[4,5]. Liver transplantation has been suggested recently for the treatment of organic acidemia (propionic aciduria, methylmalonic aciduria). In patients affected by these diseases, liver transplantation does not correct the enzyme deficiency in other organs beside the liver. Although quality of life is generally improved, patients remain at risk of severe extrahepatic disease complications[6-8]. Liver cirrhosis with severe portal hypertension develops in an about 25% of the patients affected by cystic fibrosis. Liver transplantation should be considered before the development of end-stage liver failure and when pulmonary function is still preserved (FEV_1 > 50%).

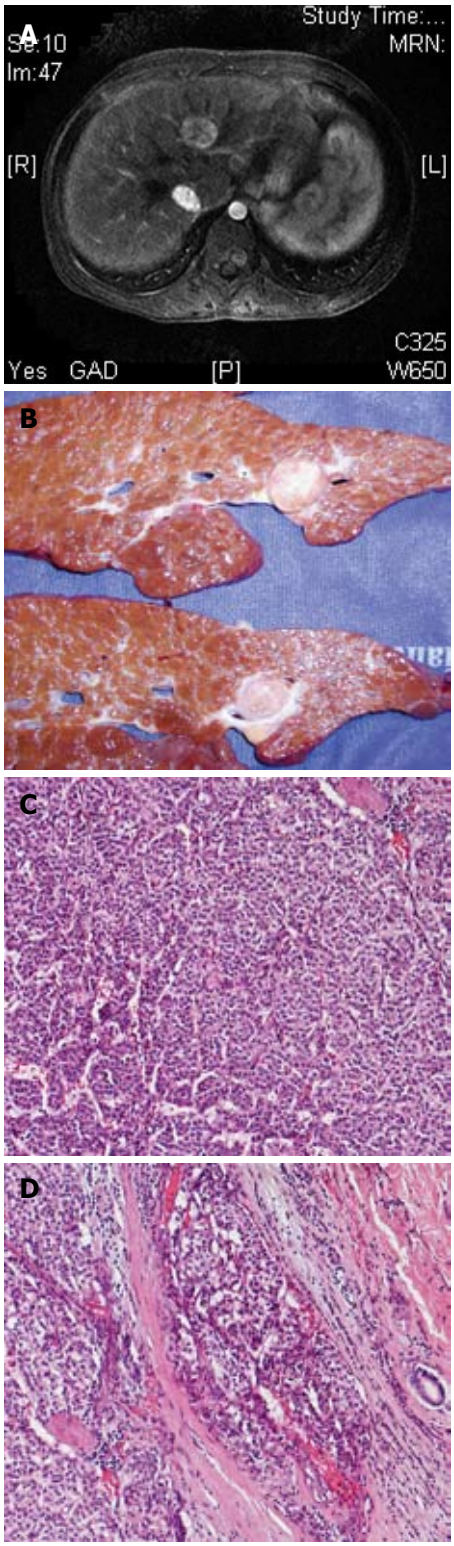

Figure 1 Adolescent affected by tyrosinemia who developed hepatocellular carcinoma, despite 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione therapy. A: Magnetic resonance imaging displays a 26-mm lesion. B: After liver transplantation, the resected liver showed multiple nodules in the left lobe. C: Histological sections from the nodule revealed hepatocellular carcinoma. D: Microvascular invasion.

Acute liver failure

Acute liver failure is a rare event in children; recovery without transplantation occurs in 15%-20% of patients with severe hepatic encephalopathy. A prospective study from the Pediatric Acute Liver Failure Study Group

Figure 2 Non-resectable hepatoblastoma.

has indicated that in 49% of patients (54% of children aged 1 year), the cause of acute liver failure cannot be determined and that total bilirubin ⩾ 5 mg/dL, international normalized ratio (INR) ⩾ 2.55 and hepatic encephalopathy are risk factors predictive of death or liver transplantation[9]. In a large retrospective United Network for Organ Sharing (UNOS) data analysis, it has been shown that 5-year patient and graft survivals of children with acute liver failure are significantly lower than the survival of children transplanted for biliary atresia (73% and 59% *vs* 89% and 78%, respectively)[10].

Liver tumors

Hepatoblastoma is the most common liver tumor in children and, when non-resectable, should be treated with total hepatectomy and liver transplantation (Figure 2). Children with hepatoblastoma should first be treated with chemotherapy and then be evaluated for resection or transplantation[11]. Hepatocellular carcinoma in children is rare and is often secondary to congenital liver disease. The development of hepatocellular carcinoma has been reported in biliary atresia, Alagille's syndrome, progressive intrahepatic cholestasis (recently also hepatoblastoma has been reported in a child with this condition). In children with tyrosinemia, there is a 33% incidence of hepatocellular carcinoma before 2 years of age that seems to be reduced if not eliminated by 2-(2-nitro-4-3 trifluoromethylbenzoyl)-1,3-cyclohexanedione (NBTC) therapy.

CONTRAINDICATIONS TO LIVER TRANSPLANTATION

Current contraindications to liver transplantation in children are: (1) non-resectable extrahepatic malignant tumor; (2) concomitant end-stage organ failure that cannot be corrected by a combined transplant; (3) uncontrolled sepsis; and (4) irreversible serious neurological damage. Whereas in adults there are limitations to access to liver transplantation waiting lists for patients with primary liver tumors, in children, the approach is much more liberal and the indication should be discussed on a case by case analysis with pediatric oncologists.

EVALUATION OF THE TRANSPLANT CANDIDATE

The primary goal of the evaluation process is to identify appropriate candidates for liver transplantation and to establish a pre-transplantation plan. The following steps are usually considered: (1) confirm the indication for transplantation; (2) determine the severity of the disease; (3) consider alternative treatments to transplantation; (4) exclude contraindications to transplantation; (5) identify active infections and assess the immunological status of the child; (6) rule out cardiac malformations that might need to be corrected before transplantation; (7) establish a pre-transplant therapeutic plan: immunizations, when possible, nutritional support to optimize growth, dental care, prevention or treatment of drug-induced side effects (e.g. osteopenia secondary to prolonged steroid intake); (8) inform parents, and the patient if possible, on the transplantation procedure and on the post-transplantation period in order to motivate and prepare them to accept and deal with all issues and possible complications of the procedure; and (9) evaluate social status and logistic issues.

PRIORITIZATION

In the early 1980s, waiting time and severity of illness expressed by patient location (home, hospital, ICU) were the primary factors used to stratify patients. Later on, it was shown that waiting time had no relationship to mortality, except for urgent acute liver failure patients, and therefore, that an allocation policy based on objective medical criteria was needed. Based on data derived from the Studies of Pediatric Liver Transplantation research group, a pediatric end-stage liver disease score (PELD) was created, using bilirubin, INR, serum albumin, age > 1 year, and growth failure to predict waiting list mortality[12]. Additional PELD points are awarded for specific risk factors not taken into account in the PELD equation, such as hepatopulmonary syndrome, metabolic diseases, and liver tumors. The adoption of the PELD score in the USA has improved the access and accountability of the allocation system. However, the PELD score has not proven to be a successful predictor of outcome following transplantation[13,14].

THE TRANSPLANT OPERATION

The first liver transplant was performed by Thomas Starzl, in 1963, on a 2-year-old child affected by biliary atresia[15]. The patient died in the operating room of uncontrolled hemorrhage. After this first case, and up to the early 1980s, the only technical option for pediatric liver transplantation was to transplant the whole liver of a donor with a weight as close as possible to that of the recipient. Given the low number of pediatric donors, up to 50% of the children on the waiting list would die before they could receive a transplant[16]. The development of techniques that allow surgeons to transplant portions of livers from adult donors has

completely changed the fate of liver transplantation in pediatric patients.

Whole-liver transplantation

The procedure of whole-liver procurement in pediatric donors can be performed exactly as in adults, applying a technique that is a combination of the initial procurement technique described by Starzl *et al*[17], and the most recently described rapid flush technique[18,19]. Whole-liver pediatric transplantation can be performed with two different techniques: the classic technique with inferior vena cava replacement, and the piggyback technique[20] with preservation of the native inferior vena cava. The present authors routinely use the classic technique in the vast majority of whole liver transplants. Veno-venous bypass is generally not used in pediatric liver transplantation, given that patients generally tolerate explantation well, provided that volume replacement has been adequate. Adopted techniques are almost identical to the ones used in adults recipients. In cases in which the liver is encased in adhesions, as it is in biliary atresia, we recommend that surgeons first approach the hepatic hilum from the right posterolateral aspect, identifying the Roux-en-Y jejunal limb, which is transected with a linear stapler or between ligatures. This allows better exposure and dissection of the hilum. If the portal vein is small and sclerotic, it has to be dissected proximally to the confluence of the splenic and superior mesenteric vein, dividing the coronary vein of the stomach. The portal vein anastomosis will then be performed by means of a donor interposition vein graft. In difficult dissections, the vena cava can be clamped above and below the liver before completing the mobilization of the liver itself.

Several methods of arterial reconstruction have been proposed. It is our preference to anastomose the small arterial vessels encountered in pediatric whole liver transplantation in an end-to-end manner by using the magnification loops (3.5 ×) and interrupted or running 8-0 polypropylene sutures. We generally do not use the branch patch technique, and in the case of aberrant arterial anatomy, the supraceliac aorta is the inflow vessel of choice. The use of arterial conduits anastomosed to the infrarenal aorta is avoided if possible.

In theory, biliary tract continuity can be restored through direct anastomosis between the new liver's hepatic duct and the recipient's common bile duct. However, the most common type of biliary reconstruction adopted in pediatric patients is hepaticojejunostomy. In biliary atresia patients, the reconstruction uses the previous Roux-en-Y limb of the hepatic portoenterostomy, if suitable; otherwise a 40-cm Roux-en-Y jejunal limb is created. The authors' attitude is not to use a T tube, because no randomized trial so far has demonstrated any advantages in using it, and there are often biliary leaks when the T tube is pulled.

Occasionally in children, abdominal-wall closure may be impossible because of the large size of the transplanted liver. This may be remedied by creating a silo on the abdominal wall such that a temporary closure can be made[21].

Reduced-size liver transplantation

This procedure was first described by Bismuth *et al*[22] and consists in the procurement of the whole liver from an adult cadaver donor, which is reduced in its size on the back-table. In the original description, a right hepatectomy was performed on the back-table: the right lobe of the liver was discharged, while the left lobe (Couinaud liver segments 1 to 4), including the vena cava, was transplanted in a child. This technique of parenchymal reduction, very seldom used today, allows surgeons to overcome differences in size between the donor and the recipient of up to four or five times[23,24].

Following these first experiences, a more aggressive reduction that allows transplanting the liver from donors with a body weight up to 12 times the recipient's was introduced. On the back-table, the graft undergoes an extended right hepatectomy, including segment 4 and the caudate lobe. The resulting left lateral segment graft comprises segments 2 and 3, without the vena cava. The graft is transplanted retaining the recipient's vena cava, anastomosing the graft left hepatic vein to the recipient's vena cava.

Reduced-size liver transplantation shows outcomes in line, if not superior, to whole-liver transplantation, and has become an essential part of the technical expertise of pediatric transplant centers[25-30] (Table 1). The development of this technique has led to almost total elimination of child mortality on the waiting list, through the utilization of an adult liver cadaver donor. Its main limitation is that it withdraws organs from the larger adult recipient pool. For this reason, after the development of living-related and split-liver transplantation, reduced-size live transplantation is used increasingly less, and should not be considered an option anymore for pediatric liver transplantation.

Living-related liver transplantation

The first description of the procedure in which segments 2 and 3 were procured from a living donor (the mother), and transplanted in a child affected by biliary duct atresia, dates back to 1988[31,32]. Living-related liver transplants soon came to account for a substantial number of pediatric cases performed in many centers throughout the world, and the only possibility for liver transplants in countries where cadaveric organ procurement was not allowed until just a few years ago[33].

Living-donor procurement involves performing a left lobectomy during which segments 2 and 3 are separated from the remaining liver, and dissecting the parenchyma along a section running to the right of the round ligament. After the parenchyma dissection, the left branch of the portal vein, the hepatic artery, and the left suprahepatic vein are quickly clamped and dissected, and the left lobe perfused on the back-table. The recipient's procedure is similar to the one described for the transplant of segments 2 and 3 from a cadaver donor, except for the fact that the arterial anastomosis can be performed only in the left branch of the hepatic artery. The branch is small and usually anastomosed directly to the recipient's hepatic artery using the operative

Table 1 Series of pediatric reduced-size liver transplantation

Series	Period	*n*	Survival (%)		ReTX (%)	Complications (%)			
			Patient	Organ		HAT	PVT	BC	PNF
Broelsch *et al*[25]	1984-1987	9	44	33	11	0	0	11	11
Otte *et al*[26]	1984-1988	42	68	54	28	7	0	NA	5
Bismuth *et al*[22]	1984-1988	14	50	44	14	7	7	14	7
Houssin *et al*[27]	1986-1989	40	75	73	-	5	5	5	5
Kalayoglu *et al*[28]	1988-1989	12	83	67	25	16	8	0	0
Esquivel *et al*[29]	1988-1990	20	81	75	12	0	3	5	0
Langnas *et al*[30]	1988-1991	29	68	65	3	7	0	20	10

ReTX: Retransplantation; HAT: Hepatic artery thrombosis; PVT: Portal vein thrombosis; BC: Biliary complication; PNF: Primary non-function; NA: Not available.

Table 2 Series of pediatric living-related liver transplantation

Series	Period	*n*	Survival (%)		ReTX (%)	Complications (%)			
			Patient	Organ		HAT	PVT	BC	PNF
Tanaka *et al*[33]	1990-1992	37	E 90 U 57	E 90 U 57	0	U 14	E 3	E 10	0
Emond *et al*[34]	1991-1992	18	94	84	16	11	6	16	0
Broelsch *et al*[35]	1991	20	85	75	20	25	20	35	0
Malagò *et al*[36]	1991-1994	36	72	72	8	2.8	3	25	-
Otte *et al*[37]	1993-1995	30	97	93	-			20	
Haberal *et al*[38]	1990-1997	19	58	58	0	5	0	0	0
Darwish *et al*[39]	1993-2002	100	94	92	3	1	14	27	0

E: Elective cases; U: Urgent cases; ReTX: Retransplantation; HAT: Hepatic artery thrombosis; PVT: Portal vein thrombosis; BC: Biliary complication; PNF: Primary non-function.

microscope.

Living-related liver transplantation has been widely debated with regard to the ethics of performing major surgery on a healthy person. The validity of this procedure is broadly recognized, and over 1200 cases have been performed worldwide, with a donor mortality and morbidity of approximately 0.2% and 10%, respectively. Morbidity relates mainly to biliary fistulas, incisional hernias, and bleeding. In the majority of cases, living-related transplants register an excellent outcome for pediatric recipients, thanks to the possibility of performing the transplant before the child's clinical condition deteriorates. Centers with most experience in this area report survival rates between 80 and 90% after 1 year[34-39] (Table 2).

Split-liver transplantation

Split-liver transplantation, as described originally by Pichlmayr, involves procuring a whole liver from a cadaver donor and dividing it into two sections along the round ligament, leaving the vascular structures for the two portions of hepatic parenchyma intact[40]. In this way, two partial organs are obtained from a single liver: the left lateral segment (segments 2 and 3), which can be transplanted in a child, and the extended right liver (segments 1 and 4-8), which can be transplanted into an adult. This procedure involves a much longer ischemia time, which, at the beginning of its adoption, led to unsatisfactory results, with a high incidence of primary dysfunction and technical complications[41-55] (Table 3). In 1994, Rogiers described a technical variation in the split-liver technique, derived from the living-related transplant experience that consisted in dividing the liver *in situ* during the procurement procedure[56]. The technique has shown outcomes comparable to those obtained with conventional techniques[57-62] (Table 4).

The donor operation

A section of the liver is made along the falciform ligament to obtain a left graft, composed of segments 2 and 3, including the left hepatic vein, the left branch of the portal vein, and the left branch of the hepatic artery, along with the common hepatic artery and the celiac tripod, and a right graft, composed of segments 1 and 4 to 8, including the vena cava, the right branch of the hepatic artery, and the portal vein along with the origin of the mesenteric and splenic veins (Figure 3).

At the beginning of the split procedure, the hepatogastric ligament is inspected to detect an accessory left hepatic artery originating from the left gastric artery, which must be preserved. When this vessel is not detected, the ligament is sectioned. The common hepatic artery is then identified and dissected from the gastroduodenal artery up to its division into the right and left hepatic arteries. The left hepatic artery is then encircled (Figure 4A). If present, branches for the fourth segment originating from the left hepatic artery should be identified and divided. The base of the round ligament is exposed by dividing the small bridge of parenchyma that connects the lower portion of segment 4 to the left lateral section of the liver. The round ligament is dissected and completely mobilized with isolation and division of its venous connections to the fourth segment. Once the round ligament is dissected, the extrahepatic portion of the left branch of the portal vein can be identified just below the left hepatic artery.

Table 3 Series of *ex situ* split-liver transplantation

Series	Year	ADU (*n*)	PED (*n*)	Urgent (%)	Patient survival (%)		Graft survival (%)		Complications (%)			
					ADU	PED	ADU	PED	HAT	PVT	BC	PNF
Pichlmayr *et al*[40]	1989	2	0	0	50	-	50	-	0	0	0	0
Bismuth *et al*[41]	1989	2	0	100	0	-	0	-	0	0	0	0
Otte *et al*[42]	1990	1	3	75	0	66	0	66	0	0	0	0
Emond *et al*[16]	1990	5	13	38	40	63	40	53	6	6	27	24
Broelsch *et al*[24]	1990	4	21	40	25	66	20	48	NA	NA	27	NA
Langnas *et al*[30]	1992	1	9	73	NA	NA	NA	NA	7	0	20	17
Houssin *et al*[43]	1993	6	10	50	83	70	83	60	13	25	25	0
Otte *et al*[44]	1994	11	18	27	NA	NA	NA	NA	10	0	17	10
Kalayoglu *et al*[45]	1996	5	7	8	100	85	80	71	8	0	17	0
Rogiers *et al*[46]	1996	5	7	44	57	100	42	100	15	0	15	0
Azoulay *et al*[47]	1996	26	1	14	80	100	76	100	15	0	22	4
Dunn *et al*[48]	1997	0	12	50		75		66	0	0	0	0
Rela *et al*[49]	1998	15	26	12	93	89	93	84	3	0	15	0
Mirza *et al*[50]	1998	10	14	58	80	78	NA	NA	8	0	8	16
Chardot *et al*[51]	1999	0	15	31	-	66	-	62	12	19	25	0
Reyes *et al*[52]	2000	13	12	66	69	66	61	50	12	0	8	NA
Deshpande *et al*[53]	2002	0	80	20	-	89	-	86	5	1	9	0
Noujaim *et al*[54]	2003	24	36	25	NA	NA	NA	NA	3	0	20	3
Oswari *et al*[55]	2005	0	30	13	-	70	-	67	2	5	7	NA

ADU: Adults; PED: Children.

Table 4 Series of *in situ* split-liver transplantation

Series	Year	ADU (*n*)	PED (*n*)	Urgent (%)	Patient survival (%)		Graft survival (%)		Complications (%)			
					ADU	PED	ADU	PED	HAT	PVT	BC	PNF
Rogiers *et al*[56]	1996	7	7	35	100	85	85	71	0	0	0	0
Goss *et al*[57]	1997	14	12	58	85	100	78	91	0	0	14	11
Busuttil *et al*[58]	1999	NA	NA	66	85	96	86	75	3	1	3	8
Ghobrial *et al*[59]	2000	51	51	49	83	78	NA	NA	2	2	NA	8
Reyes *et al*[52]	2000	NA	NA	NA	93	100	79	83	3	0	3	7
Spada *et al*[60]	2000	36	35	25	84	85	79	76	5	10	28	2
Gridelli *et al*[61]	2003	0	90	28	-	90	-	80	7	6	33	1
Yersiz *et al*[62]	2003	57	104	-	78	75	69	64	13	11	19	26

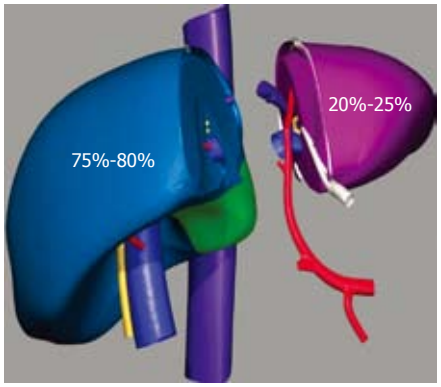

Figure 3 Spit liver allows for the procurement of two separate grafts of different size. A section of the liver is made along the falciform ligament and divides the left lateral segment from the extended right liver. The left graft, composed of segments 2 and 3, and representing 20%-25% of the total liver volume, includes the left hepatic vein, the left branch of the portal vein, and the left branch of the hepatic artery, along with the common hepatic artery and the celiac tripod. The right graft composed of segments 1 and 4-8, and representing 75%-80% of the total liver volume, includes the vena cava, the right branch of the hepatic artery, and the portal vein.

This vein must be carefully dissected and encircled (Figure 4B). The left lateral section is rotated laterally on the right side and the ligamentum venosum is dissected up to left lateral hepatic vein, which can be isolated and encircled (Figure 4C). The bile ducts of the left lateral segment are included in the porta hepatis and should not be dissected. On the contrary, the porta hepatis must be encircled and divided sharply (Figure 4D).

The section of the parenchyma can now be performed along the falciform ligament (Figure 4E). It is helpful when identifying the plane of the dissection to pass the cotton tape, which encircles the left hepatic vein on the posterior surface of the liver in the fossa of the ductus venosus, laterally to the left branch of the hepatic artery and of the portal vein (Figure 4F and G). Pulling up on this tape, the dissection of the parenchyma is usually easy. At this point, the procedure continues as a standard donor operation with heparinization, cannulation and cross-clamping of the aorta, perfusion, and cooling of the abdominal cavity. The left hepatic vein is then sectioned close to the vena cava. Care must be taken to identify a distal bifurcation of this vein. A double left hepatic vein significantly increases the technical difficulty of the implantation of the graft. In this case, the vessel should be removed with a cuff of vena cava to allow a single vascular anastomosis with

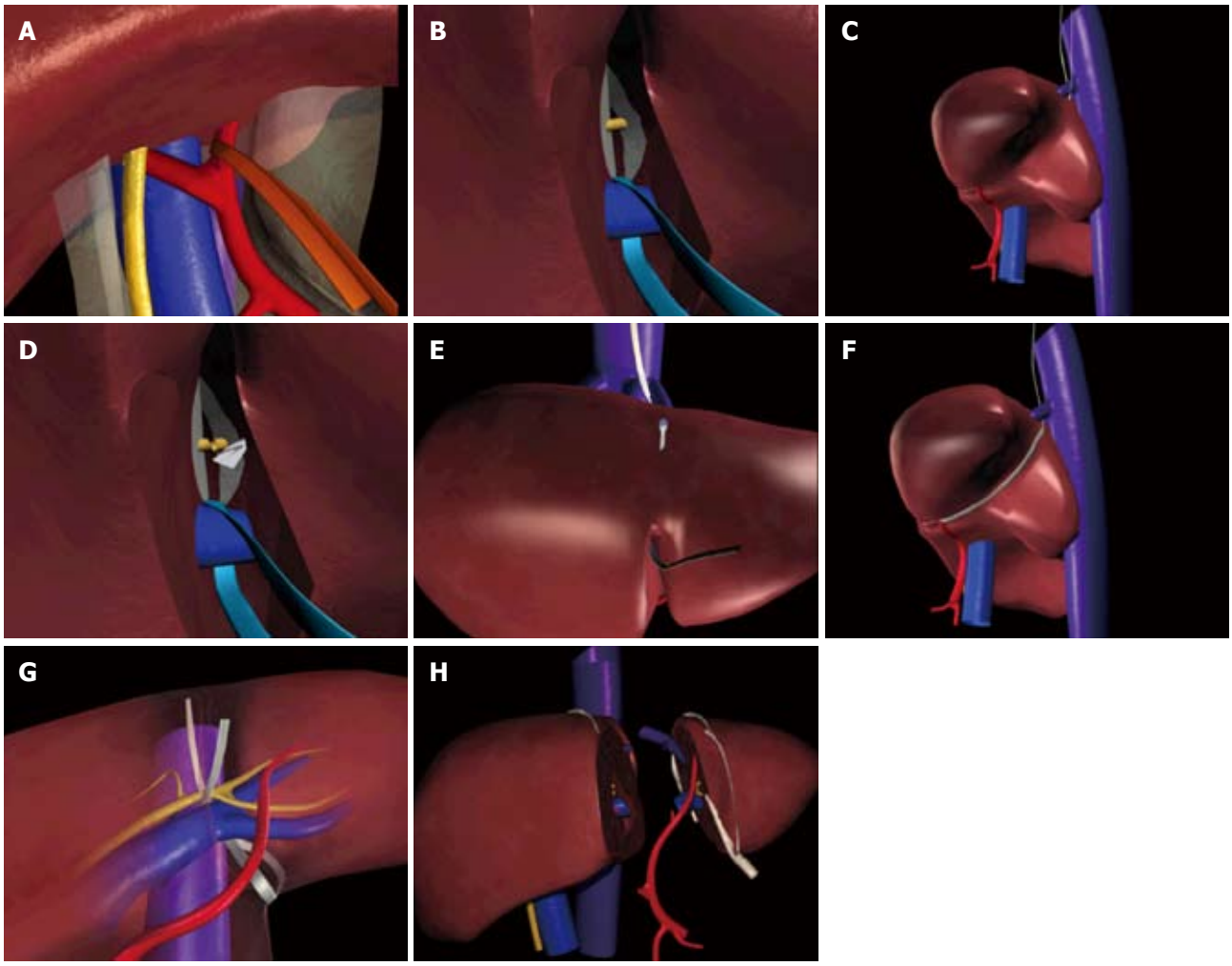

Figure 4 Main phases of split liver procurement. A: Dissection of the hepatogastric ligament and encircling of the left hepatic artery; B: Identification and encircling of the extrahepatic portion of the left branch of the portal vein; C: Isolation and encircling of the left hepatic vein; D: Division with a scalpel of the porta hepatis containing the bile duct(s) of the left lateral segment; E: Section of the parenchyma started along the falciform ligament; F: Identification of the plane of parenchymal dissection by passing the cotton tape, which encircled the left hepatic vein, on the posterior surface of the liver in the fossa of the ductus venosus; G: Laterally to the left branch of the hepatic artery and of the portal vein; H: The two partial grafts at the end of the procedure.

the recipient vena cava. The left branch of the portal vein is sectioned close to the parenchyma. The right hepatic artery is sectioned close to its origin, and the hepatic artery is dissected up to the celiac trunk, which is removed along with an aortic cuff.

The recipient operation

Recipient hepatectomy is performed, as previously described for whole-liver transplantation, with the piggy-back technique[63]. Implantation of the left lateral segment is substantially different from a whole-sized graft. Assuring an adequate venous outflow requires a careful technique of anastomosis between the left hepatic vein of the graft and the inferior vena cava of the recipient and a proper positioning of the graft itself, which is rotated clockwise 45° on a transversal plane and slightly on a frontal plane. The final position of the cut surface of the parenchyma, including the new hilum of the graft, is high and posterior, so that the portal vein and hepatic artery have a course that is curved and longer than usual.

The outflow anastomosis is end-to-side between the left hepatic vein of the graft and the inferior vena cava of the recipient, with the triangulation technique described by Emond *et al*[64]. The bridge between the ostia of the right and middle hepatic veins is cut to obtain a single opening. The ostium of the left hepatic vein may be treated in the same fashion, to obtain a further enlargement of the opening, or suture-closed. The opening is then enlarged by cutting the anterior face of the vena cava to obtain a wide reversed triangular orifice. The cuff of the left hepatic vein of the graft is trimmed as short as possible, to avoid kinking. Three 5/0 vascular monofilament sutures are placed, taking the three corners of the graft and recipient orifices (Figure 5). The graft is then placed in the hepatic fossa of the recipient and the triangular anastomosis performed with three running sutures.

The second anastomosis is the portal one, performed in an end-to-end fashion with running sutures of 6/0 or 7/0 vascular monofilament. Both the length and the section of the vessels are crucial. As already mentioned, the length should be sufficient for the vessel to make a gentle curve that reaches the hilum of the graft; as for

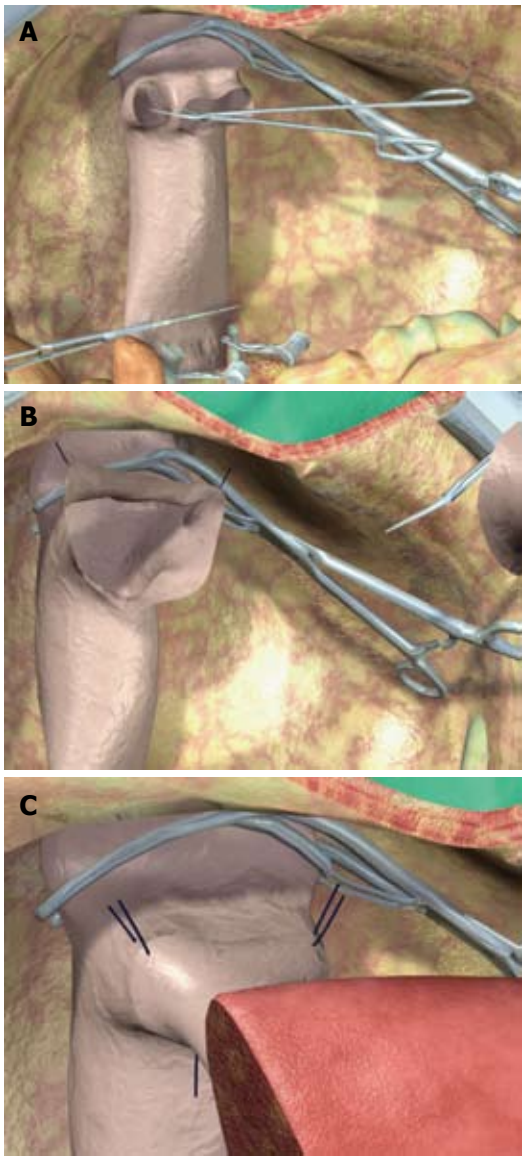

Figure 5 Anastomosis between the left hepatic vein of the graft and the inferior vena cava of the recipient, performed with the triangulation technique. A: The bridge between the ostia of the right, middle, and left hepatic veins is cut to obtain a single opening; B: The opening is further enlarged by cutting the anterior face of the vena cava to obtain a wide triangular orifice; C: Three 5/0 vascular monofilament sutures are placed, taking the three corners of the graft and recipient orifices.

the section, the limiting factor is the size of the graft cuff. In the majority of cases, the recipient's vessel matches this size rather well. If not, it can be cut at its bifurcation, to obtain a branch patch. In case of real hypoplasia of the recipient's portal vein, the confluence of the mesenteric and splenic vein can be clamped, the vessel sectioned at this level and a venous graft from the donor (usually the splenic or the external iliac vein) interposed between the confluence and the portal vein of the new liver. After completion of the anastomosis the graft is reperfused.

The arterial anastomosis comes next. The arterial axis of the graft usually includes the proper and common hepatic artery, in continuity with the celiac artery, and a patch of the aorta. The level of the anastomosis is chosen at any place along the recipient's arterial axis, and the two vessels are trimmed to obtain a similar section and an adequate length, according to what has already been stated concerning the portal vein. The anastomosis is performed end-to-end with a running suture of 7/0 or 8/0 vascular monofilament. If the recipient's arterial axis is deemed inadequate, the aorta can be clamped at the origin of the celiac artery or just below the renal arteries, and an end-to-side anastomosis can be performed at one of these sites. In the latter case, the interposition of an arterial graft from the donor, usually represented by an iliac artery, may be necessary.

The final stage is biliary reconstruction, which is always a hepaticojejunostomy with a Roux-en-Y loop. The bile duct of the graft may be single or double, although in the latter case two different anastomoses are performed (Figure 6).

Childhood hepatic malignancies have been considered a contraindication to the use of split-liver transplantation, since the need for the retention of the recipient's inferior vena cava potentially precludes obtaining a tumor-free margin[65]. A technical variation, which has allowed us and others to successfully use left lateral segment grafts to transplant children affected by hepatic malignancies, involves the replacement of the recipient's inferior vena cava using an iliac vein graft from the donor[66]. On the back-table, a wide V-shaped opening on the wall of the common iliac vein graft from the donor is made. The left hepatic vein of the left lateral segment graft is anastomosed end-to-side to the V-shaped opening on the common iliac vein with two 5/0 polypropylene running sutures (Figure 7). On the recipient, a total hepatectomy is usually performed using the standard technique of removing the liver together with the retrohepatic vena cava. At this point, the left lateral segment graft with the iliac vein graft is anastomosed to the suprahepatic vena cava in an end-to-end fashion with a 4/0 polypropylene running suture. The inferior edge of the iliac graft is then anastomosed to the infrahepatic vena cava with a 5/0 polypropylene running suture.

Donor selection

The following factors must be considered when a donor is evaluated for a specific patient.

Dimensional matching: The selection of a graft with an adequate parenchymal mass is critical to success. The minimal hepatic mass necessary for recovery is not clearly established, and its calculation must take into account the temporary loss of hepatocytes caused by the donor's injury or treatment, as well as preservation injury, acute rejection, or technical problems. Several formulas have been proposed to estimate adult and pediatric normal liver volume[67-72].

Considering that preservation injury is greater in organs from deceased donors, the hepatic mass of a graft procured from a cadaver donor should be greater than the calculated mass necessary using a living-donor liver segment. In the authors' experience, a donor weight range 20%-30% above or below that of the recipient is

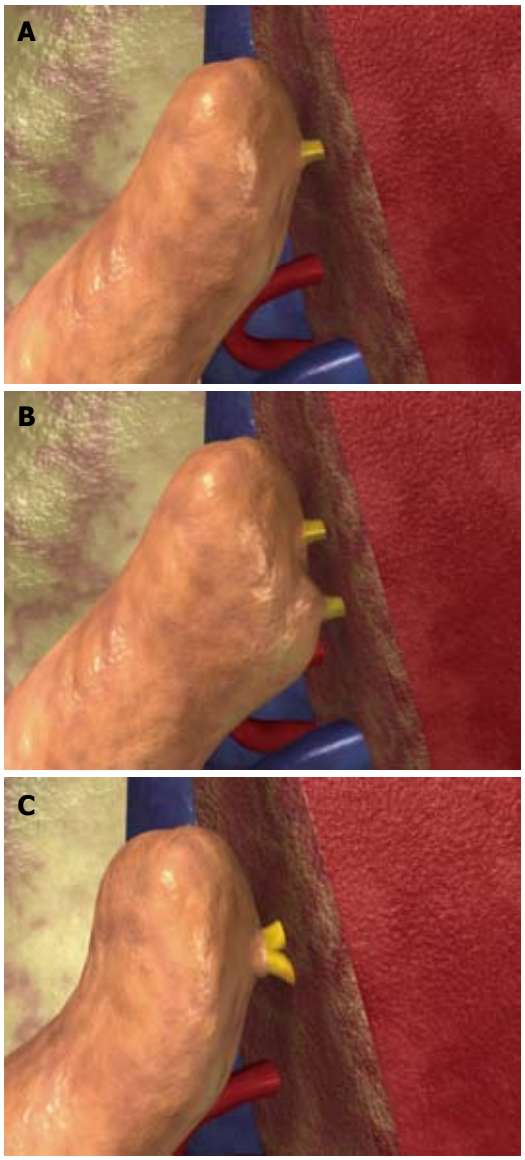

Figure 6 Biliary reconstruction performed by means of hepatico-jejunostomy. The bile duct of the graft can be single or double, although in the latter case, two different anastomoses are performed (B) or, if the two ducts are closed sufficiently, a common orifice can be created and anastomosed to the bowel loop (C).

ideal for whole-organ donors, however these values can be extended down to 50% below and 100% above, taking into consideration body habitus and factors that would increase recipient abdominal size, such as ascites and hepatosplenomegaly. When selecting donors of partial grafts, a graft fraction of 1%-3% of the recipient body mass is optimum, while a graft-to-recipient weight ratio < 0.7 is usually associated with inferior overall allograft and patient survival. In the authors' experience, a liver is procured and transplanted as a whole graft when the donor-to-recipient body weight ratio is ⩽ 2. When the donor-to-recipient body weight ratio is between 2 and 12, the graft is considered for split liver[60,61].

Donor characteristics: Donor-organ suitability is assessed by evaluating clinical information and by biochemical tests. Particular attention is paid to donor

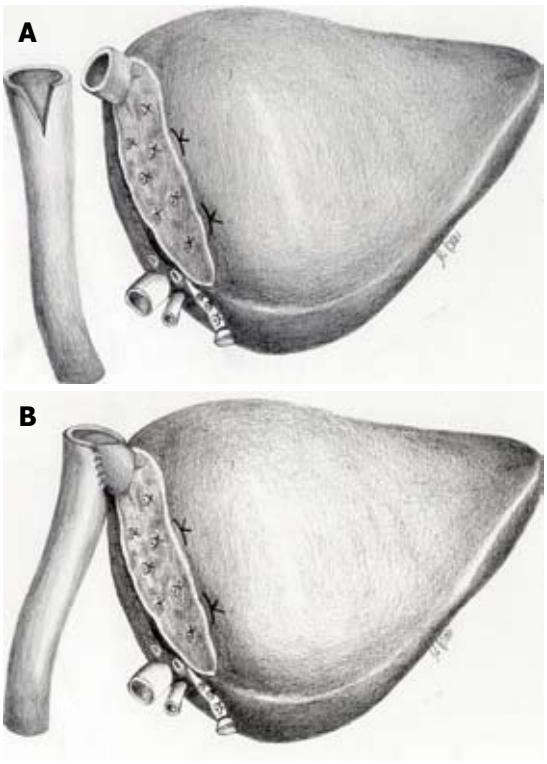

Figure 7 Use of left lateral segment grafts to transplant children affected by hepatic malignancies, with replacement of the recipient's inferior vena cava using an iliac vein graft from the donor. On the back table a wide V-shaped opening on the wall of the common iliac vein graft from the donor is made (A), and the left hepatic vein of the left lateral segment graft is anastomosed end-to-side to the V-shaped opening on the common iliac vein (B).

age, intensive care hospitalization time, infections, hemodynamic stability. Biochemical tests do not serve as good benchmarks of functional capability, even if severe electrolyte disturbances and deteriorating trends identify increased risk. In questionable cases, biopsy of the donor liver at the time of organ harvest or during evaluation of live donors is helpful to identify pre-existing liver disease or steatosis. Quite extended criteria can be used in donors of whole allografts, especially when ischemic time is limited, without compromising the outcome. On the contrary, restricted selection criteria have been proposed when split-liver transplantation is considered. Commonly accepted donor selection criteria for split-liver procurement are: (1) age 15-50 years; (2) weight > 40 kg; (3) no past history of liver dysfunction/damage; (4) liver function tests within 2-5-fold of normal values; (5) normal macroscopic appearance of the graft; and (6) hemodynamic stability[73]. Nevertheless, the authors have adopted a liberal policy of liver splitting. The decision of whether or not to split a graft is based mainly on recipient, rather than on donor, criteria. Children requiring re-transplantation or who have fulminant hepatic failure are not excluded. Donor evaluation does not require special or additional invasive or non-invasive tests. Using these extended criteria for donor selection, we have been able to transplant all the children in need with no mortality on the waiting list and good overall patient and graft survival rates[60,61].

No consistent data exist on the effect of donor age on the long-term results of pediatric liver transplantation. Data from multicenter registries have shown that pediatric patients receiving livers from pediatric-age donors have significantly better graft survival compared to those receiving livers from donors aged > 18 years[74,75]. These data strongly support the primary use of pediatric donors for pediatric recipients, but are not to be considered a contraindication to the use of adult donors in pediatric transplantation. The limited availability of pediatric donor organs does not allow us to satisfy the need of an increased waiting list population. Moreover, the results obtained using adult donors are biased by the policy to use older donors only in high-risk urgent cases. For split-liver transplantation, the authors used donors over the age of 50 years without affecting the 3-year patient and graft survival[76]. In addition, pediatric donors can be safely used for split-liver procurement and transplantation: left lateral segment is transplanted in a small child, while the extended right lobe can be used in larger children, adolescents or adults[77,78].

Living-donor selection: In living-donor transplantation, the evaluation and selection of a donor, usually a parent or first-degree relative is performed on the assumption that donor safety can be assured and that the donor's liver function is normal. Donors should be 18-55 years of age, and have an ABO-compatible blood type. Following a satisfactory medical and psychological examination by physicians who are not directly involved with the transplantation program, vascular imaging is performed to assess the hepatic arterial anatomy. Donor safety has been excellent in all living donor series.

EARLY POSTOPERATIVE PERIOD

The early postoperative period consists of managing problems related to technical complications and to the prevention, diagnosis, and treatment of acute rejection and infection episodes. Postoperative complications usually present with a combination of cholestasis, rising hepatocellular enzyme levels, and variable fever, lethargy and anorexia. This non-specific symptom complex requires specific diagnostic evaluation before establishing treatment, and empiric therapy may result in misdiagnosis, morbidity and mortality.

Primary non-function

The lack of graft functional recovery can be seen in the first hours following transplantation, with high lactate levels, increased prothrombin time and partial thromboplastin time, and failure of the patient to wake despite sedation suspension. This extremely serious complication must be treated aggressively and immediately by infusing prostaglandin E_1, adopting the necessary measures to prevent a brain edema (mannitol infusion, hyperventilation), and addressing the effects of the liver failure by infusing plasma and glucose. If the signs of lack of functional recovery persist for more than a few hours, the patient needs a new transplant as soon as possible. Lesser degrees of allograft dysfunction occur more frequently but are usually reversible. The status of the donor liver contributes significantly to the potential for primary non-function because of ischemic injury secondary to anemia, hypotension, hypoxia, or direct tissue injury. A possible cause of primary non-function is hyperacute rejection, a rare phenomenon characterized by rapid intraparenchymal vascular thrombosis, mediated by pre-formed antibodies that bind to the vascular endothelium and trigger the complement system. Antibodies are generally directed against protein alloantigens such as foreign MHC molecules or less differentiated alloantigens expressed on endothelial cells.

Vascular complications

The hepatic artery anastomosis carries the highest risk of thrombosis (5%-18%) and leads to massive graft necrosis in cases of early onset. Hepatic artery thrombosis occurs in children three to four times more frequently than in adult transplant patients, and occurs most often within the first 30 d after transplantation and in small babies transplanted with whole livers[62,79]. When hepatic artery thrombosis is identified early (Figure 8), reconstruction can be attempted to avoid allograft necrosis[80]. When allograft failure develops, urgent re-transplantation is the only option. Late thromboses (occurring some weeks after the transplant) can manifest with biliary complications (stenosis or dehiscence of the biliary anastomosis, intrahepatic bilomas) or sepsis. Rarely, allograft necrosis occurs. Stenosis of the hepatic artery usually occurs at the anastomosis and in many cases may progress to complete thrombosis. Clinical manifestations include cholestasis or graft failure caused by diminution in hepatic blood flow. Non-invasive diagnosis relies on Doppler ultrasound with calculation of resistive indices and systolic acceleration time. Treatment modalities include revision of the anastomosis or balloon angioplasty (Figure 9).

A typical complication of a left lateral segment graft is stenosis at the level of the anastomosis between the left hepatic vein of the graft and the native vena cava, which in the worst cases can lead to acute Budd-Chiari syndrome. However, since the introduction of the triangulation technique, this complication has become quite rare[68]. When present, outflow venous obstruction can be treated by cavography and balloon angioplasty (Figure 10).

Finally, portal vein thrombosis occurs in 5%-10% of recipients. It is more frequent in children transplanted for biliary atresia, because of pre-existing portal vein hypoplasia, which requires replacing the entire portal vein down to the confluence of the superior mesenteric vein with the splenic vein to avoid low-flow-related thrombosis. Early thrombosis following transplantation, detected by ultrasound screening, requires immediate anastomotic revision and thrombectomy[81]. Later thrombosis is usually detected by decreased platelet counts and increasing spleen size or gastrointestinal bleeding (Figure 11). Interventional radiographic stent

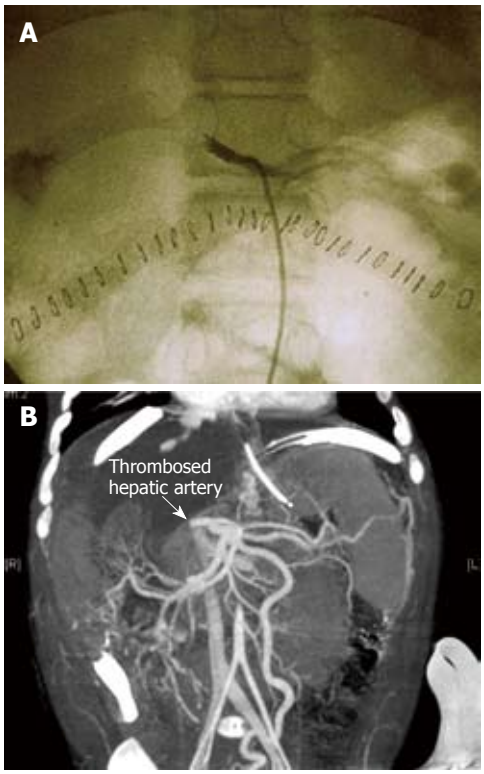

Figure 8 Selective celiac angiography showing early hepatic artery thrombosis after left lateral segment transplantation. A: Conventional angiography is the gold standard for radiographic diagnosis of hepatic artery thrombosis. B: Nowadays, the sensitivity of multiphase, multislice computed tomographic angiography with multidetector reconstruction approaches that of conventional angiography.

placement or balloon dilation has been successful in patients who have portal anastomotic stenosis but is less successful when complete thrombosis has occurred[82]. Portal venous shunting may be needed in patients who have progressive portal hypertensive complications.

Biliary complications

Biliary complications occur in approximately 10%-30% of pediatric liver transplant recipients, depending on the type of allograft used[62,83-85]. In the early postoperative period, the presence of bile-like fluid in the abdominal drainage is strongly suggestive of a bile leak. Ultrasound evidence of intrahepatic biliary ducts dilatation, elevated alkaline phosphatase and γ-glutamyl transferase (GT), and/or recurrent cholangitis suggest anastomotic or intrahepatic biliary stricture or small bowel obstruction at or distal to the Roux-en-Y anastomosis. Sometimes, non-specifically elevated liver function tests may be caused by a biliary stricture; in these cases a liver biopsy showing biliary duct proliferation and portal tract enlargement may help in differential diagnosis (Figure 12). Complications after duct-to-duct biliary reconstruction can be treated by dilation and internal stenting. With recurrent stenosis or persistent postoperative leak, Roux-en-Y choledochojejunostomy is the preferred treatment. In small children and in all patients transplanted for biliary atresia or with a partial graft, Roux-en-Y choledochojejunostomy is the reconstruction method of choice. In these patients,

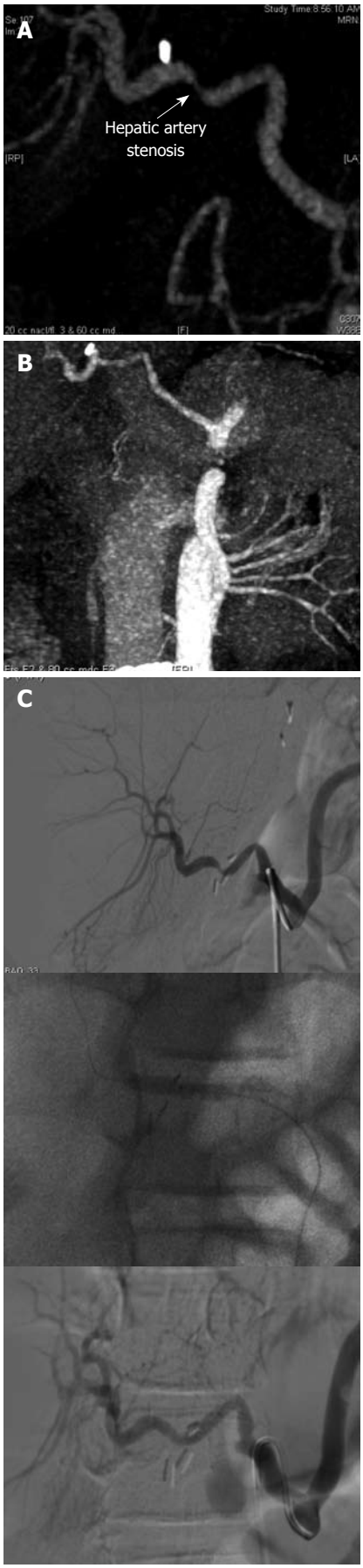

Figure 9 A case of hepatic artery stenosis. Reconstructed computed tomographic angiography demonstrating severe hepatic artery stenosis in an extended right graft recipient (A), and complete resolution of the stenosis 6 mo later (B), after stenosis treatment by early interventionally guided balloon angioplasty (C).

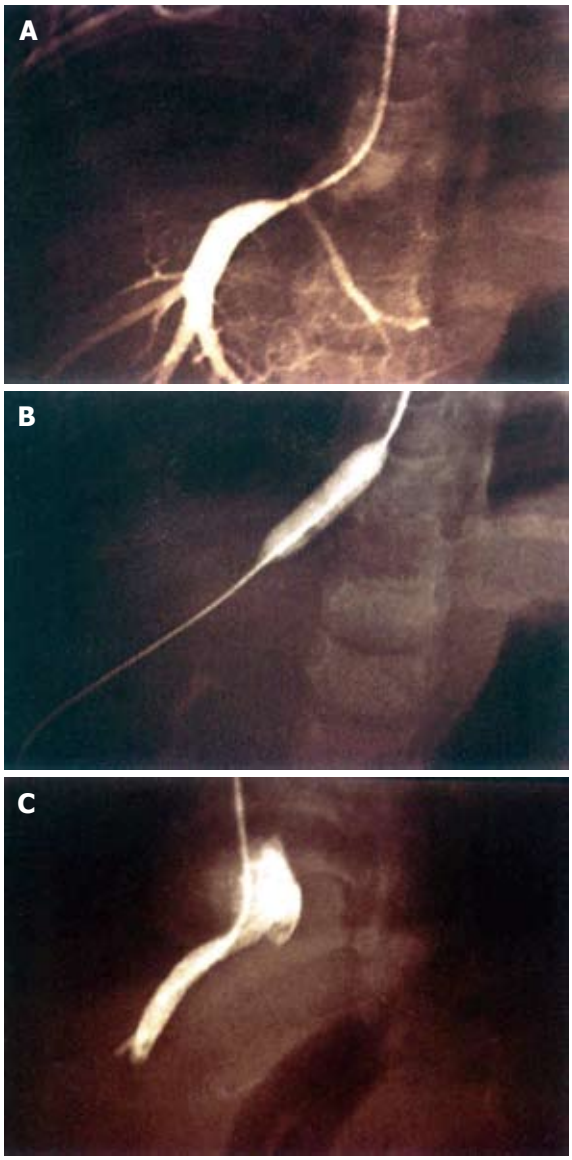

Figure 10 Venogram of hepatic venous outflow obstruction after left lateral segment split-liver transplantation. Venogram demonstrates a stenosis at the left hepatic vein anastomosis (A). Balloon angioplasty is performed (B), with resolution of the stenosis (C).

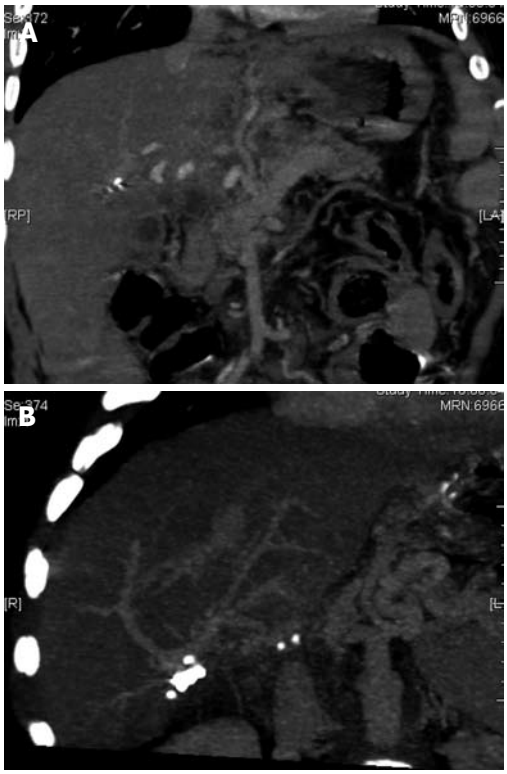

Figure 11 Portal vein thrombosis. Computed tomographic angiography with evidence of portal vein thrombosis and cavernomatous degeneration with collateral drainage through the left gastric vein (A), and evidence of intrahepatic portal flux restoration (B).

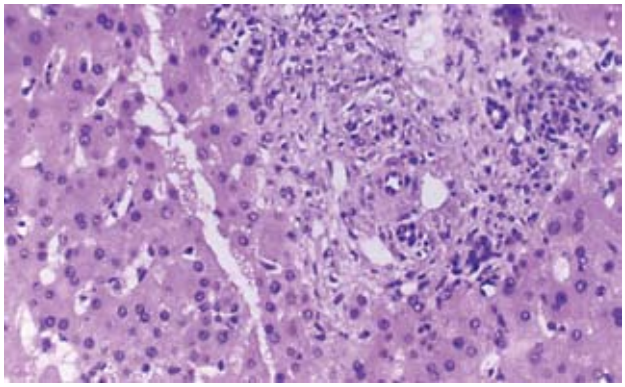

Figure 12 Liver biopsy performed in a left lateral segment recipient because of non-specifically elevated liver function tests. Histology shows biliary duct proliferation and portal tract enlargement suggestive of mechanic cholestasis.

dilatation and stenting are performed by percutaneous transhepatic cholangiography (Figure 13). The presence of multiple bile ducts has a documented increased risk for biliary complications[86].

Reoperation and re-transplantation

Early second-look reoperation is commonly used in several centers for the best diagnosis and treatment of bile leakage, hemorrhage, bowel injury secondary to multiple intra-abdominal adhesions, and sepsis. Infants and small children who have had only initial skin closure require secondary laparotomy for musculofascial closure in 5-7 d[87].

The overall incidence of re-transplantation ranges from 8% to 29%. The incidence of re-transplantation is similar for whole-organ allografts and partial allografts. The majority of re-transplantations result from acute allograft damage caused by either hepatic artery thrombosis or primary non-function; chronic rejection and biliary complications are uncommon causes. When re-transplantation for acute organ failure is undertaken in a timely manner, patient survival exceeds 80%. When re-transplantation is performed after prolonged immunosuppression for chronic allograft failure, often complicated by multiorgan insufficiency, the survival is only 50%.

Acute rejection

About 20%-50% of patients develop at least one episode of acute rejection in the first weeks after liver transplantation. The clinical picture of rejection

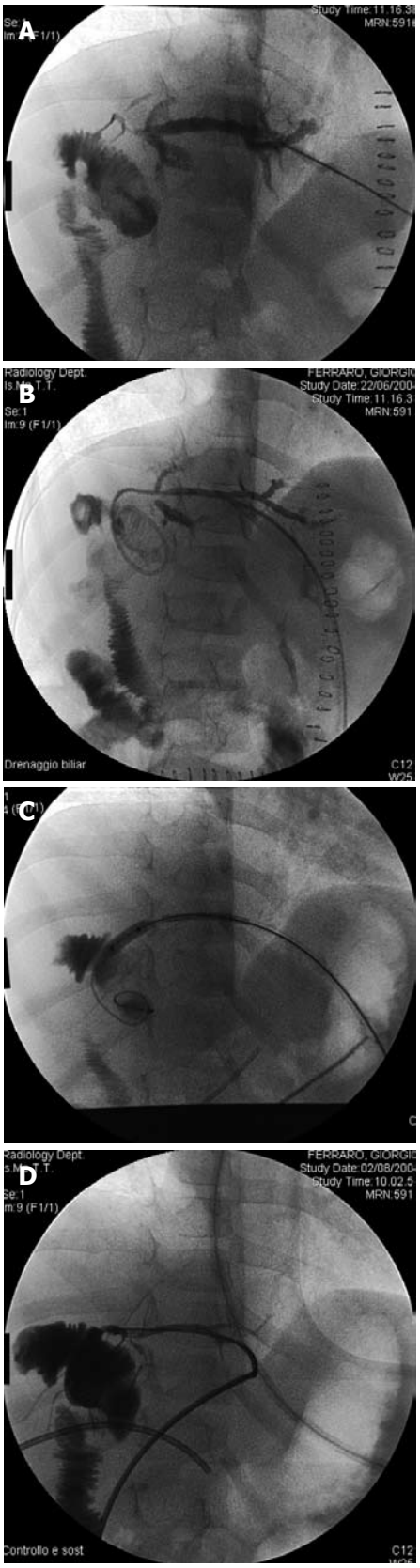

Figure 13 A case of biliary stenosis. Percutaneous transhepatic cholangiography performed in a left lateral segment recipient demonstrating intrahepatic biliary tree dilatation with stenosis of the hepaticojejunostomy (A), balloon bilioplasty (B), and transanastomotic percutaneous transhepatic biliary drainage positioning (C). Resolution of the stenosis after three sessions of bilioplasty (D).

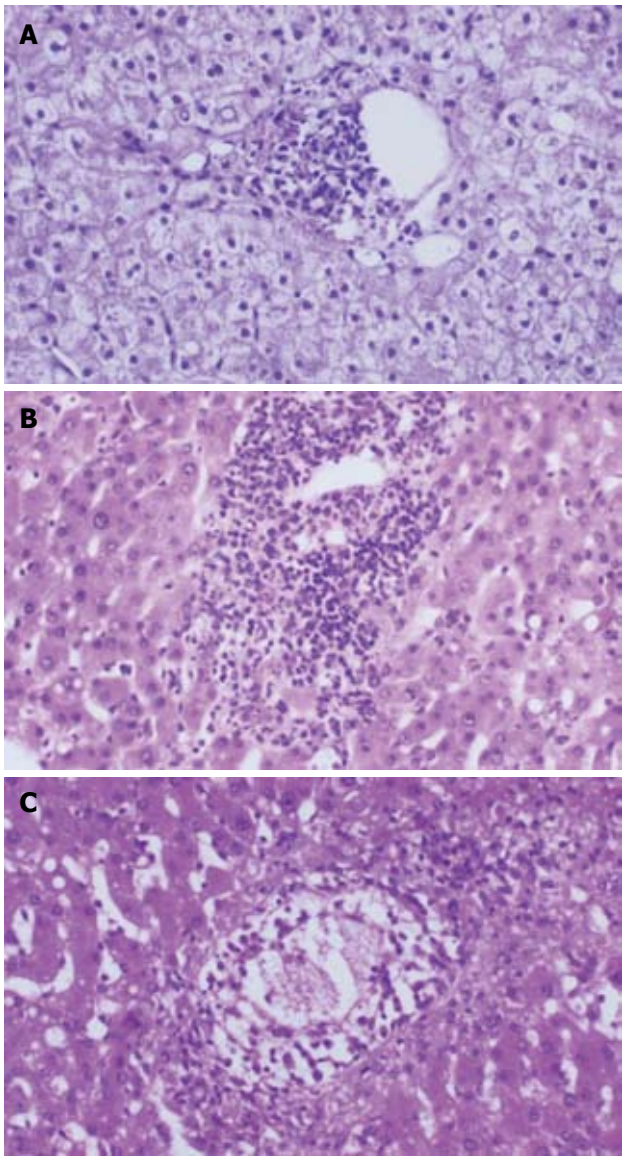

Figure 14 Acute cellular rejection: histopathological findings and grading. A: Mild acute cellular rejection, portal tracts are mildly expanded because of a predominantly mononuclear, but mixed portal inflammation. Rejection infiltrate is composed of blastic and small lymphocytes, eosinophils, macrophages, and occasional plasma cells. Lymphocytes are also present inside the basement membrane of the small bile ducts and in the subendothelial space of small portal vein branches. B: Moderate acute cellular rejection, all the portal tracts are markedly expanded by a predominantly mononuclear, but mixed inflammation. Centrilobular inflammation and hepatocyte necrosis and dropout are absent. C: evere acute cellular rejection, severe expansion of the portal tracts because of inflammation with focal portal-to-portal bridging; perivenular inflammation with hepatocyte necrosis and dropout; inflammation and damage to small bile ducts.

includes fever, irritability, malaise, leucocytosis, often with eosinophilia, and increased γ-GT, bilirubin, and transaminases. A liver biopsy is required to confirm rejection. Acute rejection is characterized by the histological triad of endothelialitis, portal triad lymphocyte infiltration with bile duct injury, and hepatic parenchymal cell damage[88] (Figure 14). Severity of acute rejection is scored according to the Banff scheme, which includes the descriptive grades indeterminate, mild, moderate, and severe, and a semi-quantitative rejection activity index (RAI) scoring on a scale from 0 to 3 the prevalence and severity of portal inflammation, bile duct damage, and subendothelial inflammation[89] (Tables 5

Table 5 Banff grading of acute liver allograft rejection

Assessment	Criteria	RAI
Indeterminate	Portal inflammatory infiltrate that fails to meet criteria for the diagnosis of acute rejection	1-2
Mild	Rejection infiltrate in a minority of the triads that is generally mild and confined within the portal spaces	3-4
Moderate	Rejection infiltrate expanding most or all of the triads	5-6
Severe	As above for moderate, with spillover into the periportal areas and moderate to severe perivenular inflammation that extends into the hepatic perenchyma and is associated with perivenular hepatocyte necrosis	> 6

Table 6 Rejection activity index (RAI)

Category	Criteria	Score
Portal inflammation	Mostly lymphocytic inflammation involving, but not noticeably expanding, a minority of the triads	1
	Expansion of most or all of the triads by a mixed infiltrate containing lymphocytes with occasional blasts, neutrophils, and eosinophils	2
	Marked expansion of most or all of the triads by a mixed infiltrate containing numerous blasts and eosinophils with inflammatory spillover into the periportal parenchyma	3
Bile duct inflammation damage	A minority of the ducts are cuffed and infiltrated by inflammatory cells and show only mild reactive changes such as an increased nuclear-to-cytoplasmatic ratio of the epithelial cells	1
	Most or all of the ducts infiltrated by inflammatory cells. More than an occasional duct shows degenerative changes such as nuclear pleomorphism, disordered polarity, and cytoplasmatic vacuolization of the epithelium	2
	As above for the 2nd criterion, with most or all of the ducts showing degenerative changes or focal luminal disruption	3
Venous endothelial inflammation	Subendothelial lymphocytic infiltration involving some, but not a majority, of the portal and/or hepatic venules	1
	Subendothelial infiltration involving most or all of the portal and/or hepatic venules	2
	As above for the 2nd criterion, with moderate or severe perivenular inflammation that extends into the perivenular parenchyma and is associated with perivenular hepatocyte necrosis	3

and 6).

The primary treatment of rejection is a short course of high-dose steroids. Bolus doses administered over a 3-6-day period with a rapid taper to baseline therapy are successful in the majority of cases. When refractory or recurrent rejection occurs, conversion from cyclosporine to tacrolimus, or antilymphocyte therapy using the monoclonal antibody, ornithine-ketoacid transaminase orthoclone, have been successfully used[90,91].

INFECTIONS

Immunosuppressive drugs used to prevent rejection inhibit activation of T lymphocytes, medullar cell proliferation and macrophage function, therefore creating an optimal environment for the development of infections. Infectious complications now represent the most common source of morbidity and mortality after transplantation.

Bacterial infections occur in the immediate post-transplantation period and are most often caused by Gram-negative enteric organisms, enterococci, or staphylococci. Sepsis originating at sites of invasive monitoring lines can be minimized by replacing or removing all of the intraoperative lines soon after transplantation. The use of prophylactic antibacterial antibiotics is discontinued as soon as possible to avoid the development of resistant organisms.

Fungal infection is a potential problem in the early post-transplantation period. To prevent fungal infection, aggressive protocols for pre-transplantation prophylaxis have been proposed[92]. Fungal infection most often occurs in high-risk patients requiring multiple operative procedures, re-transplantation, hemodialysis or continuous hemofiltration, pre-transplant chemotherapy, and multiple antibiotic courses. The authors use antifungal postoperative prophylaxis with liposomal amphotericin B only in high-risk patients undergoing liver transplantation.

Early and severe viral infections are caused by viruses of the herpes family, including Epstein-Barr virus (EBV), cytomegalovirus (CMV), and herpes simplex virus[93]. The risk of developing either CMV or EBV infection is influenced by the preoperative serological status of the transplant donor and recipient[94,95]. Seronegative recipients receiving seropositive donor organs are at greatest risk. Various prophylactic protocols, including intravenous IgG and hyperimmune anti-CMV IgG, associated with acyclovir or ganciclovir have been used to decrease the incidence of symptomatic CMV and EBV infection, although seroconversion in naive recipients inevitably occurs[94,96]. The suspicion of CMV infection is suggested by the presence of fever, leukopenia, maculopapular rash and hepatocellular abnormalities, respiratory insufficiency, or gastrointestinal hemorrhage. Hepatic biopsy or endoscopic biopsy of colonic or gastroduodenal sites allows early diagnosis with immunohistochemical recognition. Nowadays, the availability of specific antiviral drugs like ganciclovir, foscarnet and more recently valaciclovir, have radically modified the prognosis of CMV infection. At the start of the 1990s, the concept of pre-symptomatic therapy

was introduced as a strategy to prevent the incidence of CMV-related disease, based on the principle of not administering antiviral medications up to the point when these will have maximum effect, and monitoring CMV antigenemia (pp65) or viremia (CMV DNA)[97,98].

Herpes simplex virus infections, similar to those seen in non-transplant patients, require treatment with acyclovir when diagnosed.

EBV infection represents a potential risk for the pediatric transplant recipient. EBV infection has a variable clinical picture including a mononucleosis-like syndrome, hepatitis-simulating rejection, extranodal lymphoproliferative infiltration, peritonsillar or lymph node enlargement, or encephalopathy. Monitoring of EBV blood viral load by quantitative polymerase chain reaction (PCR) is the best predictor of risk. When evidence of active infection exists, an acute reduction in immunosuppression is mandatory. The authors recommend monthly EBV-DNA PCR counts and more frequent monitoring in case of increasing viral load levels. As a result of the lack of a standardized EBV DNA count methodology, no common cutoff exists. In the authors' experience, more than 500 genomes/10^5 peripheral blood leukocytes identify patients who benefit from reduction in primary immunosuppression[99]. Antiviral therapy with ganciclovir and CMV-IgG is also used, although no definitive data support their use[100,101].

Other post-transplantation infectious complications include adenovirus hepatitis, varicella, and enterovirus-induced gastroenteritis. *Pneumocystis carinii* infection has been nearly eliminated by the prophylactic administration of sulfisoxazole and trimethoprim or aerosolized pentamidine.

MANAGING IMMUNOSUPPRESSIVE THERAPY

The immune system recognizes the graft as foreign and begins a destructive immune response mediated principally by the T lymphocytes. In order to avoid destruction of the graft, immunosuppressive drugs must be administered. Progress in transplant surgery in the last 20 years has been characterized in large part by the introduction of calcineurin inhibitors that today represent the keystone of most immunosuppressive protocols[102,103]. In the last decade, new drugs that selectively target various cellular activation pathways have been proposed and used. The following are the most commonly used drugs in pediatric liver recipients.

Corticosteroids

Corticosteroids were the first drugs to be used to control rejection and are still an essential element of the immunosuppressive regimen; they are effective in both the prevention and treatment of graft rejection. They act through intracellular receptors expressed in all cells of the body. Their immunosuppressive action mechanism, not fully clarified yet, is linked to the suppression of antibody production; inhibition of synthesis of cytokines such as interleukin-2 (IL-2) and interferon-γ; reduction in the proliferation of helper and suppressor T cells, cytotoxic T cells, and B cells; and the migration and activity of neutrophils.

Long-term clinical experience with steroid use has documented a host of adverse effects. Over-immunosuppression is associated with increased incidence of bacterial, fungal and viral infections. In addition, patients taking steroids carry an increased risk for developing malignancies, especially lymphomas and skin cancers[104]. Detrimental metabolic effects of steroids are wide ranging and are of particular concern for the pediatric transplant patient[105-107]. In terms of hospital costs, the calculated 10-year cumulative expense for steroid-related complications in adult kidney recipients has been shown to be 5300 $ per patient per year[108]. Efforts are underway to develop immunotherapy regimens in which steroids can be withdrawn early, or not used at all.

The experience of steroid weaning after pediatric liver transplantation was summarized in 2000 by Reding[109]. There are a total of nine recent studies, not all of which were non-randomized and uncontrolled. Steroid treatment could be successfully stopped in 21%-100% of the transplanted patients. The risk of rejection was not significantly increased, and varied from 7% to 29%. Chronic rejection did not seem to be increased[110-118] (Table 7). The conclusions of this review are the following: (1) weaning of steroids after pediatric liver transplanatation is safe and, most of the time, beneficial; and (2) in many patients, calcineurin inhibitor monotherapy can be achieved, suggesting that the next step could be the adoption of steroid-free immunosuppressive protocols.

In a non-randomized study, Reding *et al*[119] compared pediatric liver transplantation under steroid-free immunosuppression in children who received combined tacrolimus and antibody to the IL-2 receptor of T cells (basiliximab), with matched historical recipients taking tacrolimus and steroids. Twelve-month rejection-free survival was similar in the steroid-free group compared with the corticosteroid group. The authors performed the first prospective, controlled, randomized study designed for children undergoing liver transplantation to test the possibility of avoiding the use of corticosteroids under baseline tacrolimus immunosuppression plus basiliximab induction, which confirmed no harmful effect of steroid avoidance on graft acceptance[120].

Corticosteroid withdrawal or avoidance can be difficult in patients with autoimmune hepatitis, primary biliary cirrhosis, or primary sclerosing cholangitis. In these patients it might be desirable to include steroids in the immunosuppressive protocol as a principle, although definitive and convincing data are not available.

Calcineurin inhibitors

Cyclosporine and tacrolimus are classified as calcineurin inhibitors because they inhibit T-cell responses and bind to intracellular proteins called immunophilins.

Table 7 Literature review of immunosuppressive protocol with steroid weaning after pediatric liver transplantation

Author	Year	Patients (*n*)	Protocol	Weaning (%)		Graft loss	Rejection (%)	
				Performed	Success		Acute	Chronic
Margarit *et al*[110]	1989	18	CsA+Aza	83	61	13%	27	13
Andrews *et al*[111]	1994	119	CsA+Aza[1]	44	67	No	13	No
Dunn *et al*[112]	1994	73	CsA+Aza	51	76	4%	7	4
McDiarmid *et al*[113]	1995	13	CsA+Aza			No	No	No
McKee *et al*[114]	1997	29	TAC	83	71		29	
Martin *et al*[115]	1998	55	CsA+Aza	44	76	No	11	No
Reding *et al*[109,116]	2000	375	CsA (*n* = 23)		21	No	No	No
			CsA-ME (*n* = 24)			No	No	No
			TAC (*n* = 31)			No	10	No
Atkison *et al*[117]	2002	94	CsA+Aza[2]	71	91		21	
Toyoki *et al*[118]	2004	8	TAC	100	100	No	13	No

CsA: Cyclosporine; CsA-ME: Cyclosporine microemulsion; Aza: Azathioprine; TAC: Tacrolimus. [1]In 53% of the weaned children; [2]Some patients received antilymphocyte globulin or OKT3 induction.

The immunophilin-drug complex competitively binds to and inhibits the phosphatase activity of calcineurin. Calcineurin inhibition indirectly blocks the transcription of cytokines, particularly IL-2, which regulate the proliferative T-cell response[121]. Calcineurin inhibitors have similar side-effect profiles, which include dose-dependent nephrotoxicity, neurotoxicity, and hypertension. Most adverse effects are reversible after dose reduction or discontinuation of the drug[122,123]. Tacrolimus has not been associated with cosmetic adverse effects such as hypertrichosis and gingival hyperplasia observed in cyclosporine-immunosuppressed children. Moreover, tacrolimus is associated with less hyperlipidemia and a lower adverse cardiovascular risk profile than cyclosporine[124], but with slightly more *de novo* diabetes and gastrointestinal symptoms[125]. In some studies, tacrolimus has been described to cause a higher incidence of post-transplant lymphoproliferative disease[126,127], but this has not been confirmed in other authors' experiences[128]. Hypertrophic cardiomyopathy has been reported with prolonged use of tacrolimus at unusually high levels[129].

Calcineurin inhibitors are mainly absorbed from the small intestine and are metabolized in the liver and small intestine by the cytochrome P4503A enzyme system[130]. The majority of their metabolites are excreted in bile[131]. The most important interactions are with enzymes or drugs that induce or inhibit the cytochrome P4053A, which results in reduced or increased calcineurin inhibitors levels.

Tacrolimus or cyclosporine usually represents the primary drug of most immunosuppressive regimens. Over the last 10 years, the use of tacrolimus has increased, being nowadays preferred to cyclosporine[132]. Tacrolimus and cyclosporine have been compared in large multicenter trials that showed similar 1-year patient and graft survival, with a significantly reduced incidence of acute rejection as well as steroid-resistant rejection in children treated with tacrolimus. Moreover, tacrolimus is superior to cyclosporine for the treatment of rejection episodes that may resolve when patients are switched from cyclosporine to tacrolimus therapy[97,133].

Table 8 Desired trough concentrations of calcineurin inhibitors after pediatric liver transplantation

Time post-transplant (mo)	Target level (mg/L)	
	Cyclosporine	Tacrolimus
0-3	200-250	10-15
4-12	150-200	8-10
> 12	50-100	5-8

Cyclosporine: The microemulsion form of cyclosporine, Neoral, is the formulation mainly used, which has replaced the original formulation Sandimmune because of its greater and more consistent bioavailability. Pharmacokinetics features of cyclosporine that are to be considered in children are the following: (1) cyclosporine bioavailability correlates with age, being lower in younger patients; and (2) cyclosporine is metabolized in children at a higher rate than adults, and appears to be inversely related to age[134]. The type of biliary anastomosis (e.g. Roux-en-Y biliary anastomosis for biliary atresia) and concomitant disease (e.g. cystic fibrosis) may affect absorption and bioavailability[135,136]. The recommended starting dose of Neoral is 5 mg/kg twice daily, which should be administered orally within the first 12 h of abdominal closure. Intravenous cyclosporine can be administered at a dose of 2 mg/kg per day in two divided doses by continuous infusion over 2-6 h in case of poor absorption or inadequate trough concentrations. After the first administration, the dose is adjusted in order to keep trough concentrations within a recommended target range (Table 8). Trough levels are poor predictors of rejection episodes or outcome of graft recipients[137], therefore, drug concentration in blood drawn 2 h post-dose has been proposed recently to be a superior estimate of the subsequent 12 h cyclosporine exposure[138,139].

Tacrolimus: The recommended tacrolimus starting dose is 0.05-0.1 mg/kg, administered orally within the first 12 h after abdominal closure. Subsequently, doses are adjusted in order to maintain trough concentrations

Table 9 Use of sirolimus in primary immunosuppressive regimens in liver transplantation

Author	Immunosuppression	No. of patients	Survival (%)		Acute rejection (%)	Follow-up (mo)
			Patient	Graft		
McAlister *et al*[153]	TRL, SRL, STER[1]	32	92		3	8
McAlister *et al*[154]	TRL, SRL, STER[1]	56	93	91	14	23
Peltekian *et al*[155]	TRL, SRL, STER[1]	42	93	90	10	14
Pridöhl *et al*[156]	TRL, SRL, STER	22	91	78	14	14
Sindhi *et al*[157]	TRL, early SRL, STER	6			17	15
	TRL, late SRL, ATG	9			33[2]	3

ATG: Antithymoglobulin; SRL: Sirolimus; STER: Corticosteroids; TRL: Tacrolimus; [1]Corticosteroids withdrawal 3 mo after transplantation; [2]Rejection episodes observed before sirolimus was introduced in the immunosuppressive regimen.

within a recommended target range (Table 8). The trough level is widely accepted for routine tacrolimus drug level monitoring. Large inter- and intra-individual differences in pharmacokinetics exist. The elimination half-life of tacrolimus in children is 50% of that in adults, and clearance is correspondingly two to four times faster[140,141]. Therefore, children require higher doses to achieve similar tacrolimus concentrations.

Mycophenolate mofetil

The active metabolite of mycophenolate mofetil, mycophenolic acid, is a selective inhibitor of the enzyme inosine monophosphate dehydrogenase, which is essential for the *de novo* pathway of purine synthesis[142]. Inhibition of the *de novo* pathway results in the depletion of guanosine nucleotides and arrested lymphocytes replication because they are unable to use the alternative pathway for nucleotide production[143].

Mycophenolate mofetil has been used successfully as an alternative immunosuppressive agent in patients with chronic rejection, refractory rejection, or severe calcineurin inhibitor toxicity[144,145]. Mycophenolate mofetil has also been used in calcineurin-inhibitor and corticosteroid-sparing immunosuppressive protocols, without increasing the risk of rejection[146,147]. The suggested dose for pediatric liver transplant recipients is 15 mg/kg twice daily[148]. Pharmacokinetic studies showed large inter-individual variations in mycophenolic acid parameters[149,150], which indicates the need for therapeutic drug monitoring and individualized dosing. The most relevant adverse effects of mycophenolate mofetil are dose-dependent gastrointestinal symptoms and bone marrow suppression[147,151]. Acyclovir and ganciclovir increase mycophenolic acid efficacy, whereas cholestyramine, oral antibiotics, antacids, cyclosporine, and high tacrolimus concentrations reduce its concentration[148-150].

Sirolimus

Sirolimus (rapamycin) is a macrolide antibiotic with potent immunosuppressive properties that acts by blocking T-cell activation by way of IL-2R post-receptor signal transduction[152]. Sirolimus has been used in small, uncontrolled studies in liver transplant recipients (Table 9) and reduces rate of acute rejection, when used in combination with calcineurin inhibitors, even at low doses, or facilitates early steroid withdrawal, while maintaining low rates of acute rejection[153-157].

Sirolimus has also been used as rescue treatment in chronic rejection and calcineurin inhibitor toxicity[157-159], whereas attempts to use sirolimus as a single primary immunosuppressive agent have resulted in a high rate of acute rejection[160]. Sirolimus has not yet been approved by the US Food and Drug Administration for use in liver transplantation. One trial to evaluate sirolimus in liver transplant recipients was halted because of an increased incidence of hepatic artery thrombosis. In contrast, other studies have not confirmed this finding[154,161,162], and a possible benefit of sirolimus in the prevention of coronary artery restenosis after percutaneous coronary revascularization has been described[163]. Sirolimus has shown antineoplastic activity, inhibiting angiogenesis in malignant tissue through reduction of vascular endothelial growth factor secretion, which may provide a specific indication for using of the drug in patients transplanted for primary liver malignancy[164].

Sirolimus drug interactions are similar to those of calcineurin inhibitors. It has a long half-life (40-86 h) and intra- and inter-individual variation[152,165]. Therefore, daily sirolimus monitoring is not necessary and monitoring trough level twice weekly for the first month and weekly for the next month is recommended, targeting a 5-15 mg/L range. Sirolimus levels increase during simultaneous administration of cyclosporine[166]. The most relevant dose-related side effects of sirolimus are hyperlipidemia, thrombocytopenia and leukopenia[153,157].

IL-2 receptor antibodies

T cells involved in acute rejection act by exposing activation markers such as the IL-2 receptors. Therefore, anti-IL-2 receptor therapy appears to be a promising option for specific immunosuppression. IL-2 receptor antibodies have been used primarily in children as induction agents in double or triple immunosuppression protocols. Preliminary experience in pediatric liver recipients is encouraging: pooled data from the available papers from the literature encompassed 79 patients treated with daclizumab, 165 with basiliximab, and 209 no-induction controls; incidence of acute rejection was lower in the induction groups[119,120,167-172] (Table 10).

A multicenter trial studied basiliximab pharmacokinetics and pharmacodynamics in children. It demonstrated that to achieve efficacious results, pediatric patients less than 35 kg in weight should receive two intravenous 10-mg

Table 10 Use of IL-2 receptor antibodies in primary immunosuppressive regimens in pediatric liver transplantation

Author	Immunosuppression	No. of patients	Survival (%)		Acute rejection (%)	Follow-up (mo)
			Patient	Graft		
Asensio *et al*[167]	TRL, STER	21	80	80	63	12
	TRL, STER, BAS	34	80	80	30	
Strassburg *et al*[168]	TRL, STER	12			42	28
	CSA, STER, AZA	9			66	
	CSA, STER	12			42	
	CSA, STER, BAS	21			33	
Heffron *et al*[169]	TRL, MMF, STER	20	85	88	50	24
	TRL,[2] MMF, DAC, STER	61	93	73	15	
Reding *et al*[119]	TRL, STER	20			50	12
	TRL, BAS, MMF[1]	20			25	
Ganschow *et al*[170,171]	CSA, STER	54	94		54	28-52
	CSA, STER, BAS	54	98		17	
Schuller *et al*[172]	TRL, MMF, STER	12			66	14
	TRL, MMF, DAC, STER	18			0	6
Spada *et al*[120]	TRL, STER	36	91	86	32	24
	TRL, BAS	36	87	80	12	

CSA: Cyclosporine; DAC: Daclizumab. [1]Mycophenolate mofetil was given in the first 9 patients. [2]Tacrolimus was given starting from postoperative day 7.

doses, and those weighing ⩾ 35 kg should receive two 20-mg doses of basiliximab. The first dose should be given within 6 h after organ reperfusion, and the second on day 4 after transplantation. A supplemental dose may be considered for patients with a large volume of drained ascitic fluid relative to body size[173]. For daclizumab, various different dosing regimens have been used[169,174]. A dual regimen of 1 mg/kg on days 0 and 4 provides receptor saturation for up to 21 d.

POST-TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS (PTLDS)

PTLDs are a heterogeneous group of diseases, ranging from benign lymphatic hyperplasia to lymphomas. PTLD is the most frequent tumor in children following transplantation, and occurs in the majority of the cases within the first 2 years after transplantation[175]. Late forms have usually an aggressive clinical course and severe prognosis. The development of PTLD in pediatric liver transplant recipients is favored by the intensity of the immunosuppression, its lifetime duration, and the absence of prior exposure to EBV infection in 60%-80% of patients. Risk factors for PTLD development are: (1) high total immunosuppression load; (2) EBV-naive recipients; and (3) the intensity of active viral load[176,177]. No single immunosuppressive agent has been directly related to PTLD. An important pathogenic feature favoring PTLD development is EBV infection.

Treatment of PTLD is based on the immunological cell typing and clinical presentation. Documented PTLD requires an immediate decrease or withdrawal of immunosuppression, taking into account the increased risk of organ rejection[100,101]. If a tumor expresses the B-cell marker CD20, the anti-CD20 monoclonal antibody rituximab has been successfully used. In some studies, the combination of cyclophosphamide, prednisone and rituximab has shown a response rate of 100%, with minimal toxicity[178,179]. Patients who have aggressive monoclonal malignancies have poor prognosis even with immunosuppressive reduction, acyclovir, surgery, and conventional chemotherapy or radiation therapy. Recently, autologous EBV-specific cytotoxic T-lymphocytes have proved effective in enhancing EBV-specific immune responses and reducing viral load in organ transplant recipients with active infection, and have been successfully used as first-line treatment of EBV-related PTLD[180].

LATE LIVER ALLOGRAFT DYSFUNCTION

There are several potential causes of late liver allograft dysfunction and differential diagnosis can be difficult because of overlapping clinical, serological and histopathological features. Recurrence of the native liver diseases after transplantation is a less significant problem in the pediatric population in comparison to adults. Recurrent infections and immune-based diseases are the most difficult diagnostic challenges. Most late causes of liver allograft dysfunction are detected because of abnormalities in routinely monitored liver tests; clinical signs and symptoms are much less common. When signs or symptoms do occur, liver biopsy is indicated. Common causes of late dysfunction in the pediatric population are shown in Table 11.

Late-onset acute rejection

Late-onset acute rejection may show slightly different features than typical acute rejection episodes seen early after transplantation, and is commonly characterized by: (1) predominantly mononuclear portal inflammation; (2) venous subendothelial inflammation of portal or central veins or perivenular inflammation; and (3) bile duct inflammation and damage. Late-onset acute rejection can

Table 11 Common causes of late dysfunction in the pediatric population

	Incidence at 5 yr (%)	Risk factors
Acute rejection	Variable (< 30)	Inadequate immunosuppression Treatment with immune activating drugs (e.g. interferon) History of autoimmune liver disease
Chronic rejection	-3	Inadequate immunosuppression Treatment with immune-activating drugs (e.g. interferon) Refractory acute rejection Chronic rejection in a previous failed allograft
Recurrent AIH	-30	Suboptimal immunosuppression AIH type I Severe inflammation in native liver HLA DR3 or DR4
De novo AIH	< 5	
Recurrent PBC	20-30	Tacrolimus as baseline immunosuppression Living-related donor Steroid and other immunosuppression withdrawal
Recurrent PSC	20-30	Male sex; donor-recipient gender mismatch Intact colon at time of transplantation
Idiopathic post-transplant hepatitis	5-60	

AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis.

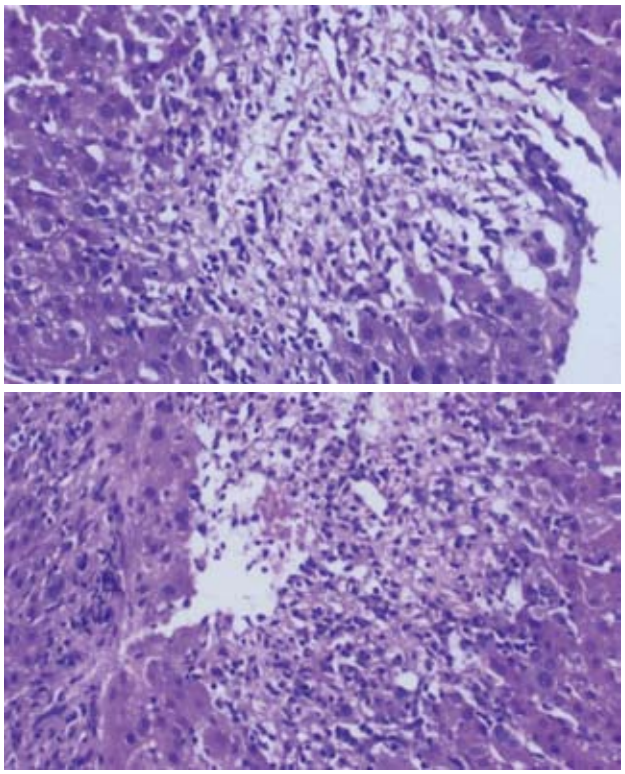

Figure 15 Histological findings in chronic rejection: Little portal inflammation in conjunction with bile duct loss affecting > 50% of the portal tracts and moderate or severe perivenular fibrosis.

also manifest as so-called central perivenulitis [181-183], or may resemble chronic hepatitis[184,185]. Mild cases may resolve spontaneously[183], but more severe forms warrant more aggressive treatment.

Chronic rejection

Chronic rejection develops in 5%-10% of transplanted patients. The primary clinical manifestation is progressive cholestasis. This course can occur within weeks from transplantation or later, and can be asymptomatic or follow persistent and/or unresponsive acute rejection and/or inadequate immunosuppression. Two clinical forms have been described[186]. In the first, named vanishing bile duct syndrome, the biliary epithelium is primarily injured with changes ranging from senescence (early stage) to severe ductopenia in at least 50% of the portal tracts (late stage)[187]. This form can be successfully treated by conversion from cyclosporine to tacrolimus immunosuppression protocols. Re-transplantation is necessary in non-responding children. The second subtype is characterized by the development of progressive ischemic injury to bile ducts and hepatocytes, which causes ductopenia and ischemic necrosis with fibrosis (Figure 15). In this setting, the diagnosis is rarely based on histology alone, because arteries with pathognomonic changes are rarely present in needle biopsy specimens. Bile duct injury and ductopenia, however, can be caused by biliary strictures, hepatic artery pathology, adverse drug reactions, and CMV. Selective hepatic angiography showing pruning of the intrahepatic arteries with poor peripheral filling and segmental narrowing supports a diagnosis of chronic rejection[188,189]. This form nearly always requires retransplantation.

Recurrent and new-onset or de novo autoimmune hepatitis

Theoretically all forms of autoimmune hepatitis after transplantation can be classified as rejection[190-192]. No conventional clinical tests differentiate an autoimmune response from rejection. The diagnosis of autoimmune hepatitis is established by a combination of serological, molecular biological and histopathological findings. Non-organ-specific autoantibodies are a requisite for the diagnosis, and they typically include smooth muscle antibodies (SMAs), antinuclear antibodies (ANAs), and antibodies to liver kidney microsome (anti-LKM)[193]. Minimal diagnostic criteria for recurrent or *de novo* au-

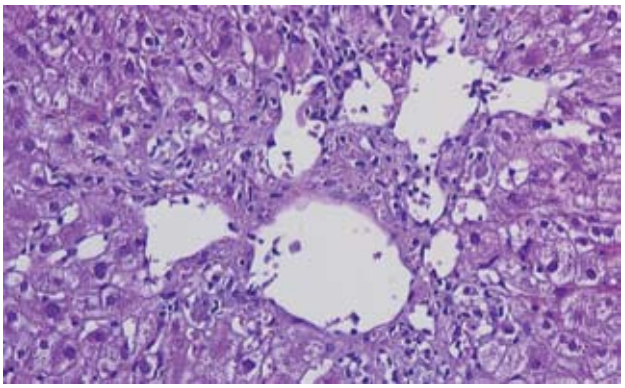

Figure 16 Histological appearance of recurrent or new-onset autoimmune hepatitis characterized by moderate portal inflammation, prominent interface activity, relative sparing of the bile ducts, and perivenular accumulation of inflammation.

toimmune hepatitis in an allograft are: (1) interface hepatitis with portal lymphocytic infiltrates (Figure 16); (2) presence of ANA, SMA or anti-LKM; (3) hypergammaglobulinemia; and (4) exclusion of virus-induced or drug-related hepatitis and late acute or chronic rejection. Most adult recipients respond to an increase in immunosuppression, whereas pediatric recipients often require the use of second-line immunosuppressive drugs (azathioprine, mycophenolate mofetil). A cautious approach to withdrawal of immunosuppression is warranted in all patients transplanted for autoimmune hepatitis, and the consequences of recurrent disease within the graft will require prolonged follow-up. A recent study, evaluating protocol liver biopsies performed in asymptomatic children 1, 5 and 10 years after transplantation, documented that chronic hepatitis is a common finding in children after liver transplantation, and is associated with a high risk of developing progressive fibrosis, which leads to cirrhosis, and with the presence of autoantibodies[194].

Idiopathic post-transplant hepatitis

Chronic hepatitis that cannot be ascribed to a particular cause is defined as idiopathic post-transplant hepatitis. Cases presenting with central perivenulitis probably represent centrilobular-based acute rejection or autoimmune hepatitis, if autoantibodies are also present[185], because allograft dysfunction usually responds to increased immunosuppression[185,195]. Some cases may represent a form of rejection with features of chronic hepatitis[195]. A diagnosis of idiopathic post-transplant hepatitis does not usually require treatment with increased immunosuppression. However, as some cases do show progressive fibrosis, the management of those with moderate to marked activity needs to be clarified.

Primary sclerosing cholangitis

Recurrent primary sclerosing cholangitis is nearly identical to that seen in native livers[196,197]. Most patients with suspected recurrent disease are asymptomatic after transplantation. An accurate diagnosis of primary sclerosing cholangitis recurrence requires well-defined cholangiographic and histological criteria. Other disorders that can produce biliary strictures after transplantation should be excluded. Graft with primary sclerosing cholangitis recurrence shows biliary strictures, acute and chronic pericholangitis, and centrilobular hepatocanalicular cholestasis periductal fibrosis[198].

Table 12 UNOS pediatric liver Kaplan-Meier patient and graft survival rates for transplants performed between 1997 and 2004

Recipient age (yr)	Patient survival (yr)			Graft survival (yr)		
	1	3	5	1	3	5
< 1	89	82	78	81	70	63
1-5	86	80	77	78	71	67
6-10	91	86	86	84	76	75
11-17	93	87	81	87	77	67

One-year survival based on 2002-2004 transplants, 3-year survival based on 1999-2002 transplants, 5-year survival based on 1997-2000 transplants.

OUTCOME FOLLOWING TRANSPLANTATION

The overall results following liver transplantation are rewarding. The European Liver Transplantation Registry (ELTR) reports liver transplantation activity in Europe, and represents 5895 children transplanted between 1988 and 2005. Overall 1-year patient and graft survival was 84% and 73%, respectively, in patients older than 2 years at the time of transplantation, and 81% and 71%, respectively, in children < 2 years of age. Ten-year patient and graft survival rates for the same age groups were 75% and 61%, and 74% and 60%, respectively. Similarly, UNOS reported survival rates of the 9064 pediatric patients transplanted between 1997 and 2004. One-, 3- and 5-year patient and graft survival rates stratified according to recipient age at the time of transplant are reported in Table 12. Overall 1-year patient and allograft survival reported to the Studies of Pediatric Liver Transplantation (SPLIT) registry, representing 1611 patients, reached 88% and 82%, respectively, while these were 83% and 74%, respectively, 4 years after transplantation. Specific factors influencing early survival include age, diagnosis, severity of illness, and possibly allograft type[199].

Age

Survival for infants < 1 year of age or weighing < 10 kg has been reported to be between 65% and 80% overall, an improvement over the previously reported rates of 50%-60%[200]. Experienced programs have described even better patient survival rates at 3 mo[201]. Improved survival in these recipients results from technical innovations, better graft preparation and avoidance of life- and graft-threatening complications such as hepatic artery thrombosis and primary non-function.

Diagnosis

Survival after transplantation is similar in patients who have cholestatic and metabolic liver disease. Early survival rates are worse for patients who have acute liver failure[9,202,203] and liver tumors[11], but their long-term survival rates are similar to those of other recipients. Asso-

ciated multiorgan failure and a limited organ-acquisition time frame influence this result. Similar decreased survival trends are seen in patients who have a PELD score > 20, in status 1 recipients, and in patients whose PELD scores deteriorate significantly before transplantation[204].

Graft type

Donor factors influencing patient and graft survival include a donor age < 6 mo or > 50 years, even if some studies have demonstrated that elderly donors can be used safely[76]. The impact on the outcome of graft type (whole, reduced, split, or living-donor) is less clear. In the SPLIT registry, recipients of whole organs had better patient and graft survival than recipients of reduced, split, or living-donor allografts[205]. The US Scientific Registry of Transplant Patients database review has reported significantly lower risk of graft failure for patients aged < 2 years who received living-donor grafts compared to whole- and split-liver recipients. Older recipients showed a higher risk of graft loss and mortality after living-donor transplantation[206]. These conflicting results may have been influenced by the diverse experience accumulated in the transplant centers. Reports of whole-organ, living-donor, and split-liver outcomes from experienced centers showed no difference in patient and graft survival, and in biliary and vascular complications[53,60-62,207,208]. Successful transplantation of very small recipients with monosegments has been reported[209]. Overall, the best results can be achieved at centers that have extensive experience with all age groups and allograft types, allowing transplantation according to the needs of the recipient. The most important prognostic factor is the severity of the patient's illness at the time of transplantation[210]. The good survival rates obtained in patients receiving living-donor transplantation are positively influenced by the possibility to schedule transplantation before the development of life-threatening complications or severe malnutrition[211]. Children with acute liver failure, PELD > 20, and severe growth retardation have significantly lower overall survival than other groups. Previous major surgery influences the incidence of complications, especially bowel perforation, but do not negatively impact overall patient or graft survival. Long-term survival is mainly influenced by the consequences of prolonged immunosuppression such as infection, PTLD, renal insufficiency, hypertension, diabetes mellitus, and coronary artery disease[212].

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TOPIC HIGHLIGHT

Salvatore Gruttadauria, MD, Associate Professor, *Series Editor*

Imaging in liver transplantation

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Abstract

The aim of this study was to illustrate the role of non-invasive imaging tools such as ultrasonography, multi-detector row computed tomography, and magnetic resonance imaging in the evaluation of pediatric and adult liver recipients and potential liver donors, and in the detection of potential complications arising from liver transplantation.

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Key words: Complications; Liver donor; Liver transplantation; Magnetic resonance; Multi detector computed tomography

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INTRODUCTION

In liver transplantation (LT) candidates, the goal of imaging is to evaluate the intra- and extra-hepatic anatomy, identify conditions that can complicate LT, and stage the neoplastic disease.

Preoperative assessment of potential living liver donors requires the evaluation of liver parenchyma to identify steatosis or lesions; the accurate evaluation of intra and extrahepatic biliary and vascular anatomy to identify congenital variants and, overall, to detect the dominant arterial branch to segment 4 to prevent accidental removal at surgery; and an accurate estimation of the volume of both liver lobes to exclude complications related to graft volume [small-for-size grafts or large-for-size grafts, characterized by a graft-to-recipient weight ratio (GRWR) less than 1%, and more than 3%, respectively].

In the post-transplant period, the goal of imaging is to identify vascular and biliary complications. The long-term follow-up also allows clinicians to identify recurrence of the primary disease and/or detect disease related to long-term immunosuppression.

In the pediatric recipient a wide spectrum of diffuse and focal diseases are indications for LT. Biliary atresia represents at least 50% of all pediatric transplantation, while the most common hepatic malignancy leading to LT is hepatoblastoma[1-5]. Other indications for LT in pediatric patients are Alagille syndrome, cystic fibrosis, tyrosinemia type 1 (associated with a high risk of hepatocellular carcinoma (HCC) development), Wilson's disease, Langerhans' cell histiocytosis, HCC, infantile hepatic hemangioendothelioma type Ⅱ (IHE), and hemangiomatosis[5-10].

In the adult recipient the most common indication for LT is still hepatitis-related liver cirrhosis with or without HCC. HCC is the fifth most common neoplasm in the world, and the third most common cause of cancer-related death. In adults it typically occurs within the cirrhotic liver. Non-alcoholic post-hepatitic cirrhosis is the most common association, but any condition that causes cirrhosis may potentially lead to HCC, including conditions such as inborn errors of metabolism. Exposure to chemical carcinogens can also cause the development of HCC. LT based on the Milan criteria is considered the optimal treatment for HCC, especially in patients with underlying chronic liver disease, because it offers a potential cure for both HCC and the underlying

liver disease. The prognosis of cirrhotic patients depends on the occurrence and progression of HCC. α-feto protein (AFP) alone shows low predictivity and sensitivity for the screening of HCC, and so imaging plays a key role in the surveillance of the cirrhotic patient[11-14].

Other focal diseases that require LT in adult patients are metastasis of neuroendocrine tumors, adenomatosis, giant angiomas, hepatic epitheloid hemangioendothelioma, and cholangiocarcinoma in selected cases, while the most common diffuse liver diseases in adults that require LT are primary biliary cirrhosis, primary sclerosing cholangitis, polycystic liver disease, Caroli's disease and Budd-Chiari Syndrome[15]. Fulminant hepatic failure can occur in both pediatric and adult patients[16].

RADIOLOGICAL ASSESSMENT OF LIVER TRANSPLANT RECIPIENTS

Ultrasonography (US)

US is usually the first imaging modality in the evaluation of a transplant candidate, independent of the disease, because it is easy to perform, widely available, relatively inexpensive, and is cost effective. US can detect morphological changes in the liver, hepatic focal lesions (cystic or solid) or abdominal masses, and signs of portal hypertension such as hypersplenism, perihepatic or perisplenic varices, and ascites.

US plays a specific role in the diagnosis of biliary atresia and in the screening of HCC in cirrhotic patients[17-20].

In biliary atresia, US may show the absence, or reduction in size, of the gallbladder and the presence of a triangular cord (TC) sign (thickness of 4 mm or more of the anterior wall of the right portal vein, seen near the portal bifurcation). The identification of a TC sign results in a sensitivity of 80%, a specificity of 98%, a positive predictive value of 94%, a negative predictive value of 94%, and an accuracy of 94% for diagnosing biliary atresia[17].

US is the most common examination for the screening of HCC in cirrhotic patients, usually performed at either 3-, 6- or 12-mo intervals, although the sensitivity and specificity reported in the literature show a wide heterogeneity, ranging from 58% to 89%, and from 75% to 94%, respectively[18-20].

On gray-scale US, HCC is predominantly hypoechoic and sometimes isoechoic, with a thin hypoechoic halo corresponding to the tumor capsule. In diffuse HCC, there is subtle disruption of the normal echo pattern, with anechoic areas due to necrosis. Color Doppler and power Doppler modes permit a real-time evaluation of the hemodynamics in liver tumors. There are, however, many limitations that can affect the assessment of tumor hemodynamics[21].

For the diagnosis of HCC, contrast-enhanced US (CEUS) is recommended by the European Association for the Study of the Liver (EASL) as the modality for evaluation of the vascularity of hepatic nodules in cirrhotic patients. Two dynamic imaging studies that show arterial hypervascularity and washout in the portal venous phase for diagnosis of HCC ranging from 1 cm to 2 cm in diameter are required. For a mass greater than 2 cm, the coincident findings of characteristic arterial vascularization that is seen on at least two imaging techniques, or hypervascularity in one imaging technique associated with washout in the portal venous and/or delayed phase, may be used to confidently establish the diagnosis without biopsy[22-26].

However, there is currently no indication for the use of microbubble contrast agents to increase the detection rate of HCC in patients undergoing US surveillance. In fact, with CEUS one can only observe the perfusion in selected lesions identified with other imaging modalities, and not in the whole liver[23-25].

US also shows a high sensitivity and specificity for excluding portal vein thrombosis (PVT). In patients with hypervascular tumors such as HCC, it is important to establish the nature of the thrombus because tumoral vascular invasion worsens prognosis and may result in exclusion from the LT program. The presence of pulsatile arterial signals inside the thrombus at color Doppler ultrasound (CDUS) is reported to be a highly sensitive and specific sign of malignant PVT[27]. CEUS, using sulfur hexafluoride microbubbles (SonoVue, Bracco SpA), seems to increase sensitivity (88%) and accuracy (92.5%) when distinguishing between benign and malignant PVT[28].

Multi-detector row computed tomography (MDCT)

In liver recipients, MDCT provides important information about liver morphology (normal or cirrhotic), intrahepatic and extrahepatic malignancy, venous benign and/or malignant thrombosis, patency of main portal vein, portosystemic collateral due to portal hypertension (spleno-renal spontaneous shunt, gastroesophageal and/or paracaval varices, and paraumbilical and caput medusae), celiac stenosis, splenic artery aneurysm, congenital arterial variants, patency, and anomalies of the inferior vena cava[29]. These findings may influence the decision to transplant, or the surgical planning of arterial and venous reconstruction. In addition, combined arterial, portal venous, and delayed-phase imaging improves the sensitivity of MDCT in detecting hypervascular neoplasms such as HCC or neuroendocrine metastases, and can also detect other tumors that enhance in a delayed phase, such as cholangi-ocarcinoma[30-33].

In pediatric candidates, the studies are usually performed with and without isosmolar or lower osmolar contrast media intravenous (c.m.i.v.) injection (Iodixanol 320 mgI/mL, Optiray 320, respectively) at a dose of 1.5 mL/kg, and at a rate that depends on the age of the patient (0.5-4 mL/s). When needed, the patient is anesthetized with intravenous propofol (0.5-1 mg/kg), without intubation. Images of the liver are acquired in the cranium-caudal direction, with slice thickness 1.25 mm or 0.625 mm, collimation 2.5 mm and table

speed 7.5 mm per gantry rotation. Usually only three phases are acquired: unenhanced phase, arterial phase, and portal venous phase. Postprocessing of the dataset offers a variety of advanced three-dimensional models of the hepatic artery and vein using multi-planar reconstruction (MPR), maximum intensity projection (MIP), and volume rendering (VR) reconstructions. The volume of the liver is usually calculated, using dedicated software, in pediatric recipients as a guide in the donor-to-dimensional matching[34,35].

In adult candidates, MDCT studies are usually performed with and without iodinated c.m.i.v. with a dose ranging from 1.5 mL/kg to 1.8 mL/kg of body weight, at a rate of 4-5 mL/s. Images of the liver are acquired in the cranium-caudal direction, during a single breath-hold acquisition, with slice thickness 1.25 mm or 0.625 mm, collimation 2.5 mm and table speed 7.5 mm per gantry rotation. A triple or quadruple-phase protocol is used: unenhanced phase, arterial phase, and portal venous phase, without and with late phase, respectively. Before the study, patients receive 500 mL of water as an oral contrast agent. Bolus tracking or test bolus technique (10 mL of contrast material at 5 mL/s) is used to calculate the correct time of the arterial phase. The portal venous phase and late phase acquisitions are generally obtained after 60 s and 180 s from the beginning of contrast injection, respectively. MIP and MPR reconstructions are usually made[30-33].

Magnetic resonance imaging (MRI)

MRI is a non-invasive and sensitive technique that is devoid of ionizing radiation. For this reason, MRI is the preferred modality in the assessment of pediatric recipients. MRI examinations are usually performed using a head coil for small infants, or body coils for larger children. All images are acquired in the axial plane in breath-hold, or with suspended respiration if under general anesthesia. If necessary, contrast medium is injected. Using Gadobenato dimeglumina 0.1 mL/kg (Bracco, SpA) it is possible to obtain information about perfusion of the liver, changes in the parenchyma and the vasculature related to cirrhosis and portal hypertension (PVT, varices, ascites), and to detect vascular congenital anomalies. In addition, the multiphasic contrast enhancement study can detect malignancy in the liver, and locoregional involvement.

Mangafodipir trisodium (MnDPDP, Teslascan, GE) is a contrast agent composed of a water-soluble chelate complex salt that is between a paramagnetic manganese (Mn^{2+}) ion and the ligand dipyridoxyl diphosphate, a vitamin B6 analogue; 50%-60% of the contrast administered is excreted through the gastrointestinal tract. For this reason, Teslascan has recently been used for the early diagnosis of biliary atresia, based on the absence of the bowel excretion of contrast material[36].

In adult recipients, especially in cirrhotic patients, MRI plays a role in detecting and differentiating HCC from other regenerative or dysplastic nodules, because it is more sensitive than multiphasic contrast-enhanced MDCT. However, it is still unclear whether MRI is more sensitive than MDCT in detecting HCC[19,20,26].

MR cholangio-pancreatography (MRCP) using single shot fast spin echo (SSFSE) single and multisection, parallel and radial acquisition can well depict disease (such as sclerosing cholangitis, Caroli's disease) of the biliary tree in adults, while in pediatric recipients, it is limited by the small caliber of the duct, thus rarely visible in neonates. In biliary atresia, it can help to demonstrate the absence of the gallbladder.

RADIOLOGICAL ASSESSMENT OF POTENTIAL LIVER DONORS

US

US is usually the first imaging modality for the evaluation of potential donors because it can identify hepatic lesions, obtain important information on the anatomy of the great vessels, such as hepatic veins and portal system, and evaluate the presence of steatosis. Due to a lack of accepted methods for quantification of steatosis on imaging, in many hospitals a biopsy is incorporated in the work-up, while in other centers, a biopsy is performed only in cases of suspicion based on clinical or imaging grounds[37].

MDCT

MDCT is the most important tool in the assessment of potential donors. MDCT can precisely depict congenital variants, if present, that can influence the surgical technique, identify focal lesions (hemangiomas, focal nodular hyperplasia, adenomas) or diffuse liver diseases (steatosis, hemocromatosis), and calculate the volume of the two liver lobes.

Congenital arterial variants are frequent, and are found in approximately 45% of donors. The identification of the dominant arterial branch to segment 4 is very important because its integrity is indispensable for the regeneration of the residual left hemiliver. This artery usually arises from the left hepatic artery (LHA), while in 25% of cases it arises from the right hepatic artery (RHA) or from both the LHA and RHA (Figure 1).

Anatomical variants of the portal system occur in 20% of the donor population; although the anomalies are not a contraindication to surgery, they must be known because they may require multiple portal anastomoses during the implantation of the right lobe into the recipient (Figure 2).

Identifying the hepatic venous anatomy is a fundamental step because it determines the hepatectomy plane that runs 1 cm to the right of the middle hepatic vein (MHV). Both accessory hepatic veins of the right inferior lobe (68% of the donor population), and large branching veins (> 5 mm) draining into the MHV from the right lobe require separate anastomosis to prevent venous congestion in the graft[37-41] (Figure 3).

Accurate volume of both liver lobes needs to be estimated to ensure that the hepatic mass is adequate for both liver donor and recipient.

MDCT scan studies are performed with and without

Figure 1 MDCT. VR reconstruction images. A: 35-year-old male, potential living liver donor. Normal anatomy of hepatic artery. CHA: Common hepatic artery; GDA: Gastroduodenal artery; PHA: Proper hepatic artery; LHA: Left hepatic artery; RHA: Right hepatic artery; S4: Artery to segment 4; B: 29-year-old female, potential living liver donor. Early bifurcation of hepatic artery. Two large arterial branches to segment 4 arising from LHA and RHA; C: 25-year-old male, potential living liver donor. LHA arising from left gastric artery. The artery to segment 4 arising from the gastroduodenal artery.

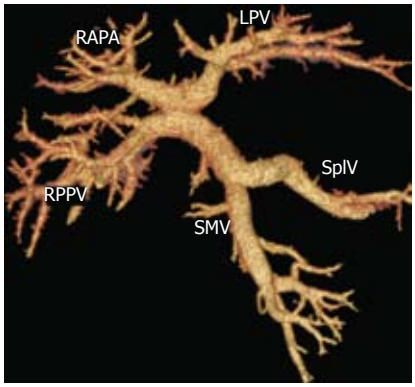

Figure 2 Thirty-two-year-old male, potential living liver donor. VR reconstruction shows a right anterior branch arising from left portal branch. SMV: Superior mesenteric vein; SplV: Splenic vein; LPV: Left portal vein; RAPD: Right anterior portal vein; RPPV: Right posterior portal vein.

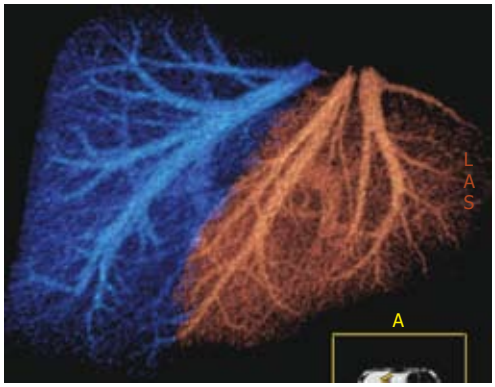

Figure 3 Forty-one-year-old male, potential living liver donor. VR reconstruction shows normal anatomy of hepatic veins. The right lobe and right hepatic vein are blue, the left lobe and the MHV and left hepatic veins are red. A cut-plane runs 1 cm to the right of the MHV.

iodinated c.m.i.v. at a dose ranging from 1.5 mL/kg to 1.8 mL/kg of body weight, and at a rate of 4-5 mL/s. Images of the liver are acquired in the cranium-caudal direction, during a single breath-hold acquisition, with slice thickness 1.25 mm or 0.625 mm, collimation 2.5 mm and table speed 7.5 mm per gantry rotation. Before the study, patients receive 500 mL of water as an oral contrast agent. Usually, a triple-phase protocol is used: unenhanced phase, arterial phase, and portal venous phase. Bolus tracking or test bolus technique (10 mL of contrast material at 4/5 mL per second) is used to calculate the correct time of the arterial phase. The peak enhancement plus 2 s is deemed as the start of the arterial acquisition to depict the arterial system. The portal venous phase is generally taken 70 s after the contrast agent has been injected to determine the exact delineation of the portal and hepatic veins. MIP and VR image reconstruction of the artery and portal venous system are usually created in the post-processing stage. The portal-venous acquisition is used for the volumetric evaluation, using dedicated software, in the postprocessing of the right and left lobe[37-39].

Some authors have proposed an all-in-one protocol to depict the biliary system, using a biliary contrast agent (Biliscopin; Schering, Berlin, Germany). However, a high incidence of adverse reactions to the biliary contrast agent, ranging from mild and self-resolving to severe systemic adverse reactions (shock-syndrome and death), has been observed[38].

Magnetic resonance cholangio-pancreatography (MRCP)

MRCP is currently considered the primary imaging tool for biliary anatomy evaluation in potential living liver donors. In fact, it is performed with new generation units equipped with high performance gradient and phased-array coils, allowing for high quality heavily T2-weighted images with increased spatial resolution in a few seconds or with respiratory triggering, eliminating most motion-related artifacts.

Only 57% of donors have a conventional biliary anatomy (Figure 4). Although the congenital variants of biliary anatomy do not represent a contraindication to liver donation, they must be identified before surgery to prevent ligation of major branches of the right lobe in the recipient and/or of the liver lobe in the donor. Multiple biliary anastomoses during the implantation of the right lobe into the recipient can be required to avoid atrophy due to biliary obstruction.

Improvements in hepatocyte-specific contrast

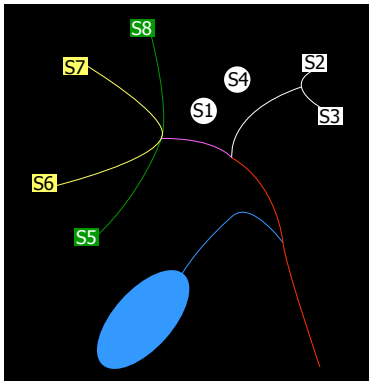

Figure 4 Normal anatomy of biliary drainage: Right posterior duct (RPD) and right anterior duct (RAD) drain, respectively, S6-S7 and S8-S5; right hepatic duct (RHD) is formed by confluence of RPD and RAD. Left hepatic duct (LHD) drains S2-S3. S1-S4 can be drained by LHD or by RHD. The common hepatic duct (CHD) arises from the confluence of RHD and LHD.

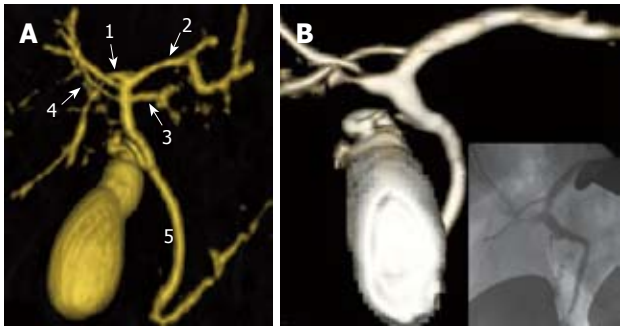

Figure 5 MRCP. VR reconstruction images. A: Twenty-two-year-old male, potential living liver donor. VR image using MRCP acquisition shows congenital anomalies of biliary drainage: RPD (1) draining in LHD (2), accessory LHD (3) and accessory RPD (4) draining in MHD (5); B: 24-year-old female, potential living liver donor. VR image after Teslascan injection shows congenital anomalies of biliary drainage: RPD draining in LHD. The finding is confirmed with intraoperative cholangiogram.

agents with biliary excretion (mangafodipir trisodium and gadobenate dimeglumine) seem to have increased the accuracy of MRI in depicting the biliary system[41,42] (Figure 5).

Some studies propose MRI as a single imaging modality for the preoperative assessment of potential donors to depict the arterial, portal and venous anatomy using MR-angiography with 3D sequence after the administration of extracellular c.m.i.v. However, MR-angiography can rarely depict the artery supplying segment 4[40,43].

RADIOLOGICAL ASSESSMENT OF POST-TRANSPLANT COMPLICATIONS

US

US and CDUS are the most important tools in the follow-up of LT patients because they show high sensitivity and specificity in detecting vascular complications.

During transplantation, CDUS is usually performed to detect the intraparenchymal flows (arterial, portal and venous), and to evaluate the velocity of flow and

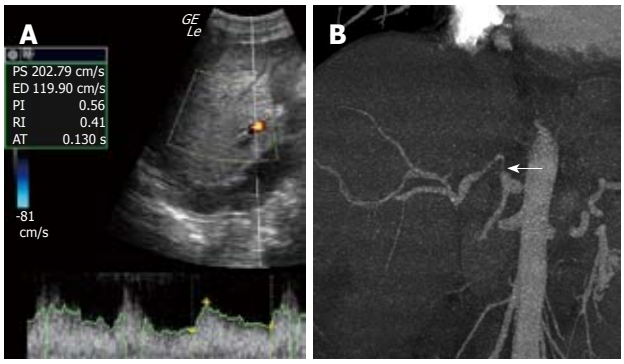

Figure 6 Fifteen-year-old male underwent orthotopic LT (OLT) for biliary atresia. A: Ten days after OLT, CDUS shows a pathologic resistance index (< 0.5) and pathologic acceleration time (> 0.08), strongly suspicious for stenosis; B: AngioMDCT confirms a stenosis of the hepatic artery after the anastomosis (arrow).

waveform to detect very early complications such as hyperacute hepatic artery or PVT[44].

After LT, CDUS is usually performed once a day during the first week, and once a week in the following 2 mo, and is key in the suspicion or identification of vascular or biliary complications.

Hepatic artery thrombosis (HAT) occurs more frequently in pediatric recipients (9%-42%) than in adult recipients (4%-12%). It frequently leads to graft failure, due to biliary wall necrosis with bilomas, biliary leakage, and hepatic infarction[45].

CDUS is able to identify up to 92% of cases of HAT, demonstrating the absence of flow in the common hepatic artery and in the intrahepatic branches. In younger subjects, in the event of complete hepatic artery thrombosis, intrahepatic flow can be sustained by small collateral neoformed vessels from the superior mesenteric artery. In these cases, the flow has a *tardus parvus* waveform. In adults, the formation of collateral vessels is almost never sufficient to prevent ischemic biliary complications. Ultrasound findings can be false positive if the hepatic artery is small or stenotic, if the flow is very slow, or if there is coexistent systemic hypotension. If US does not show an arterial flow, administration of contrast media (microbubble) can help to improve the flow visualization in the HA, differentiating between thrombosis and a patent artery in patients without HA flow on conventional Doppler US[46-49].

Hepatic artery stenosis is reported in 5%-10% of transplant recipients and can be anastomotic (in 70% of cases), perianastomotic or intrahepatic. It is most frequently caused by an error in surgical technique or by arterial damage during explantation. Near the stenosis, the Doppler ultrasound shows a focal velocity greater than 2 mL/s, and turbulence; more distally, it detects a *tardus parvus* arterial waveform with a resistance index lower than 0.5 and a systolic acceleration time (between the end of the diastole and the first systolic peak) greater than 0.08 s[46-49] (Figures 6 and 7).

Post-transplant PVT is extremely rare in adults. In children, particularly with biliary atresia, post-transplant PVT, although not usual, is not rare. The underlying

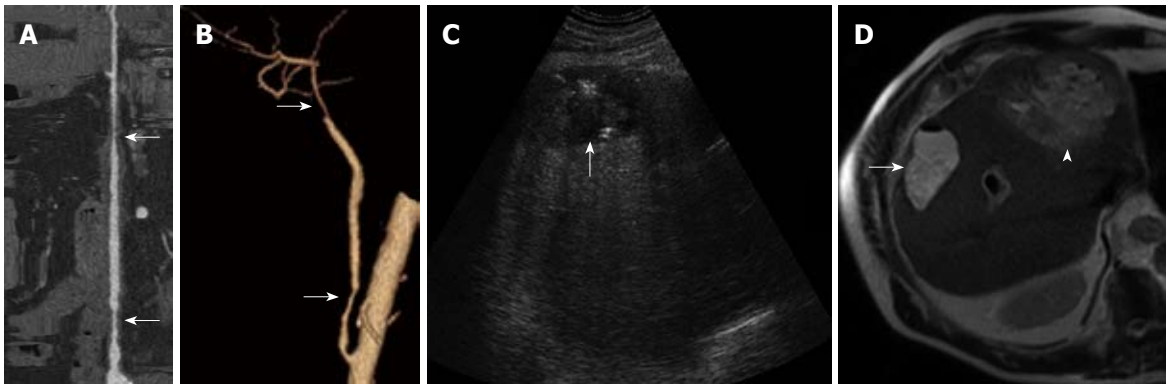

Figure 7 Fifty-four-year-old male underwent living related liver transplant (LRLT) for HCC in HCV-related cirrhosis. A and B: 3 mo after LRLT, a lumen stripe reconstruction (A) and VR reconstruction (B) show irregularities (arrow) of aortohepatic by-pass. Six months after LRLT, the patient was admitted to hospital with fever; C: US shows a hypoechoic and inhomogeneous lesion in the right lobe (arrow); D: MR T2W images show a hyperintense lesion, confirming an abscess in the right lobe (arrow). The left lobe appears inhomogeneously and diffusely hyperintense, showing a large abscess (head of arrow).

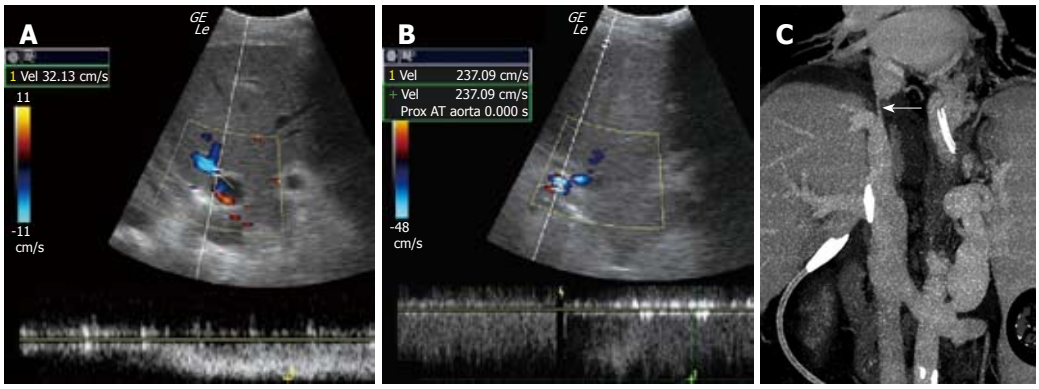

Figure 8 Fifty-one-year-old male underwent OLT for HCC in HCV-related cirrhosis. A and B: Eight days after OLT, CDUS shows a high velocity gradient between the subhepatic (A) and suprahepatic (B) segments of the inferior vena cava, with a velocity of 32 cm/s and 237 cm/s, respectively, strongly suspicious for stenosis; C: MDCT with MPR image confirms stenosis of the suprahepatic segment of the inferior vena cava (arrow).

problems in small children are not only due to smaller caliber vessels but also due to hypoplastic and sclerotic vessels brought about by pre-transplant recurrent cholangitis[50]. Color Doppler ultrasound shows the absence of flow in the portal vein, and whether it is anechogenic (recent thrombosis) or echogenic (old thrombosis). If the thrombosis is recent, it can be treated with local thrombolysis and mechanical thrombectomy.

Portal vein stenosis is more frequent in partial liver transplants than in whole liver transplants. The suspicion of stenosis arises if color Doppler shows a turbulent flow with focal aliasing in the stenotic tract, and a velocity gradient of > 4-fold[46-49].

Post-liver transplant stenosis of the inferior vena cava is rare and generally secondary to technical issues. CDUS shows an increased trans-anastomotic velocity gradient (> 4 times) and the loss of the tracing's normal phasicity[44,46,49] (Figure 8).

In partial liver transplants, hepatic vein stenosis is a frequent complication. CDUS of the hepatic veins reveals a slow (< 10 cm/s) and monophasic flow[44,46,49].

All the vascular complications described, when suspected on US and CDUS, need confirmation with contrast-enhanced MDCT, contrast-enhanced MR or with angiography.

US shows low diagnostic accuracy in identifying biliary complications, particularly in early anastomotic stenosis without a significant intrahepatic biliary duct dilatation. US is, however, an accurate tool for evaluating necrosis, bilomas, or abscess of the graft.

MDCT

MDCT angiography is the best option for confirming the ultrasonographic suspicion of early and late vascular complications (HAT, main portal vein or inferior vena cava (IVC) stenosis or thrombosis)[51]. In addition, it permits a good assessment of liver parenchyma and other abdominal organs, and the evaluation of bilomas (Figure 9), bleeding, abdominal or hepatic abscesses (Figure 7), adrenal infarction, and intestinal perforation or obstruction. MDCT can identify biliary duct dilatation, even if the anastomosis is not easy to depict.

MDCT also plays a key role in detecting late complications, such as recurrence of the primary disease, post-transplant lymphoproliferative disease (PTLD), Kaposi's sarcoma or other malignancies related to long-term immunosuppression.

MRI

MRCP after LT is the modality of choice for the

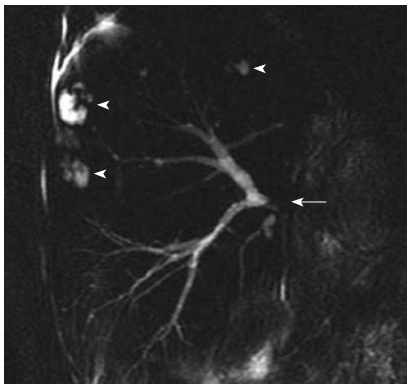

Figure 9 Sixty-one-year-old male underwent OLT for primary biliary cirrhosis, with bilio-enteric anastomosis. MRCP shows a severe anastomotic stricture (arrow) and multiple intrahepatic biliary leakages (head of arrows).

diagnosis and management of biliary complications, and shows a sensitivity ranging from 87.5% to 95.3%, a positive predictive value ranging from 92.3% to 97.6%, and an accuracy ranging from 90.4% to 95.2%[52-54]. The T2W images easily identify fluid collection (bilomas, bile leakage, biliary duct dilatation) that appear strongly hyperintense, while MRCP using SSFSE single and multisection, parallel and radial acquisition can finely depict filling defects, anastomotic or non-anastomotic stenosis, or irregularities of the biliary duct.

Bile duct complications in the various series vary from 7% to 50%. In partial liver transplants, biliary complications are more frequent because the diameters of the bile ducts to be anastomosed are smaller, and multiple biliary anastomoses are often necessary. Most of these complications occur within the first 3 mo, even though biliary stenoses and gallstones can occur months and years after transplantation.

Bile extravasation has an incidence ranging from 5% to 19%, and may occur in the T-tube insertion site, in the region of the anastomosis, or intrahepatically.

Intrahepatic bile leakage (biloma) entails the suspicion of bile duct necrosis secondary to HAT. In these cases, a new transplant is almost always necessary. However, the percutaneous drainage of a biloma can prevent sepsis and increase the likelihood of graft survival. In partial liver transplants, bile can also leak from a large bile duct damaged at the time of liver "splitting," or from the surface of the resection margin, which exposes thousands of small bile ducts (Figure 9).

Stenoses can be classified as anastomotic or non-anastomotic. Anastomotic stenoses are the result of postoperative fibroses or of errors associated with surgical technique (Figure 9). Non-anastomotic stenoses can be intra- or extrahepatic, single or multiple, and are often due to ischemic damage. In these cases, it is necessary to assess the patency of the hepatic artery. Rarely are they due to prolonged graft ischemia time, chronic rejection or cytomegalovirus infections. In transplants related to sclerosing cholangitis, intrahepatic stenosis can indicate disease recurrence.

Rare causes of bile duct obstruction are the dislocation/obstruction of the T-tube, biliary sludge, gallstones or an excessive choledochus length after the choledochocholedochostomy. The mucocele of the residual cystic duct can cause bile duct stenosis resulting from extrinsic compression.

Enhancement with mangafodipir trisodium improves the performance of MRCP for the detection and exclusion of biliary abnormalities after orthotopic LT[55].

CONCLUSION

Imaging plays a primary role in LT. It is used in the assessment of the recipient, the assessment of the potential living liver donor, and the detection of early and late complications. US, MDCT and MRI have different roles, depending on accuracy, in depicting the different goal in each period of the orthotopic LT.

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TOPIC HIGHLIGHT

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Interventional radiology procedures in adult patients who underwent liver transplantation

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Abstract

Interventional radiology has acquired a key role in every liver transplantation (LT) program by treating the majority of vascular and non-vascular post-transplant complications, improving graft and patient survival and avoiding, in the majority of cases, surgical revision and/or re-transplantation. The aim of this paper is to review indications, technical consideration, results achievable and potential complications of interventional radiology procedures after deceased donor LT and living related adult LT.

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Key words: Liver transplantation; Interventional radiology; Complication; Review; Liver

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INTRODUCTION

Liver transplantation (LT) in patients with end-stage liver diseases has become an accepted treatment. Advances in the field of percutaneous, radiological, minimally invasive techniques have increased the importance of interventional radiology in the management of patients after LT[1]. In this article, we discuss the possible applications of interventional radiology in the management of adult recipients after deceased donor LT or living related LT (LRLT), including diagnosis of graft disease, treatment of vascular complications and treatment of biliary complications. Techniques used, results and possible complications of interventional radiology procedures are described by reviewing our experience and other protocols present in literature.

DIAGNOSIS OF GRAFT DISEASE

Random liver biopsy is frequently requested after LT. Any alteration of liver function tests, not explained by diagnostic imaging, requires a liver biopsy to exclude rejection and/or other pathologies. Liver biopsy can be performed by a percutaneous approach (blind or ultrasound guided) or, in selected patients, with a transjugular approach. In our practice, in patients without ascites, percutaneous core liver biopsies are performed with an 18-Ga needle under ultrasound guide to avoid entering the bowel or other adjacent organs, and to avoid the perforation of main intrahepatic vascular structures. If coagulation defects are present (platelets < 50 000 mm^3 and/or prothrombin activity < 50%) patients receive infusion of platelets and/or fresh frozen plasma. No antibiotic prophylaxis is performed before the procedure. Complications are

infrequent, most of them being minor (pain, decrease in hematocrit value not necessitating treatment). Possible major complications of percutaneous liver biopsies are bleeding, emobilia, arterio-portal fistula and infections, and are reported in up to 3% of cases[2]. Controlled studies have shown that blind percutaneous biopsy carries a higher risk for major complications compared to ultrasound guided liver biopsy[3]. Although it has been reported by Little *et al*[2] that the presence of perihepatic ascites does not statistically affect the major or minor complications rate of image-guided percutaneous hepatic biopsy, in our center, the transjugular approach is preferred if perihepatic ascites are present. The transjugular approach is considered mandatory if a severe coagulopathy and/or massive amounts of perihepatic ascites are present. This technique reduces the risk of hemorrhage, because a biopsy specimen is acquired through the hepatic vein and any bleeding from the puncture site remains in the vascular space. In addition, if a clinical suspicion of portal hypertension is present, hepatic vein pressure gradient (HVPG) can be measured during the same procedure. The right internal jugular vein approach is usually preferred, but in selected cases, if the right jugular vein is not usable (thrombosis or difficult catheterization), the left internal jugular vein can be used[4]. In our experience, there are no major complications related to transjugular liver biopsies performed in adult liver transplant recipients. Despite transjugular liver biopsy being effective and safe for patients with contraindications to percutaneous liver biopsy, in patients with small liver or in patient with partial LT (from a living donor or deceased donor), subcapsular or intraperitoneal bleeding due to accidental perforation of the capsule is possible. Hemobilia, accidental puncture of kidney, transient dysrhythmias, and hematoma at the puncture site are the most common complications reported in other studies[5-7].

TREATMENT OF VASCULAR COMPLICATIONS

Vascular complications following LT are associated with high morbidity, graft lost and mortality rate. The majority of vascular complications develop within 3 mo of the transplant and possible complications should be considered in any LT patient with alteration of liver function tests. The clinical presentation of vascular complications is often indistinguishable from other post-transplantation complications (biliary complications, rejection, graft dysfunction, infections). Color Doppler ultrasonography (US), multidetector-row computed tomography (MDCT), and magnetic resonance are useful for diagnosis. Vascular complications can affect the hepatic artery, hepatic vein, portal vein and inferior vena cava (IVC).

Hepatic artery thrombosis (HAT)

HAT is a dramatic, potentially life-threatening, complication of LT occurring in 3%-9% of adult liver-transplanted patients[8,9]. Risk factors for HAT are considered to be surgical technique, small donor vessels, slow flow secondary to hepatic artery stenosis (HAS), ischemia-reperfusion injury, coagulation abnormalities, ABO blood group incompatible transplantation, use of aortic jumping graft and multiple rejection episodes. Ischemia caused by HAT results in severe biliary and parenchyma damage and is associated with high rates of graft loss and mortality. Urgent thrombectomy and revascularization or re-transplantation is currently considered the treatment of choice in case of early diagnosis of HAT[10]. If Doppler US and/or computed tomograghy (CT) suspicious of HAT are present, an arteriogram is usually performed to confirm the imaging finding and, in very early diagnosis of HAT, to try to restore the hepatic flow with selective thrombolytic therapy and eventual treatment of concomitant HAS with balloon angioplasty and/or stent placement. In our practice, selective catheterization of the hepatic artery stump is performed with a microcatheter and an infusion of thrombolytic drugs. In selected patients with concomitant steal syndrome from splenic and/or gastroduodenal arteries, percutaneous splenic and/or gastroduodenal artery embolization can be performed in the same session in order to increase the flow in the hepatic artery (Figure 1). Surgical thrombectomy and/or re-transplantation should be reserved for cases in which percutaneous techniques fail. Saad *et al*[11] reported successful in re-establishment of arterial

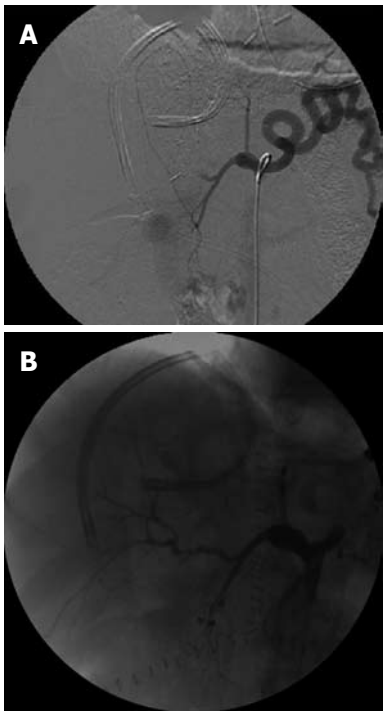

Figure 1 Status post right lobe LRLT in 18-year-old male. A: Celiac arteriogram showed acute HAT. Local thrombolysis with TPA was performed by placing a microcatheter in the hepatic artery stump; B: Celiac arteriogram after TPA infusion showed patent hepatic artery. In this patient, splenic artery embolization was performed by coils and PVA in order to increase the flow in hepatic artery. Although a good and early flow restoration was achieved, the patient underwent re-transplantation 2 d later for graft failure.

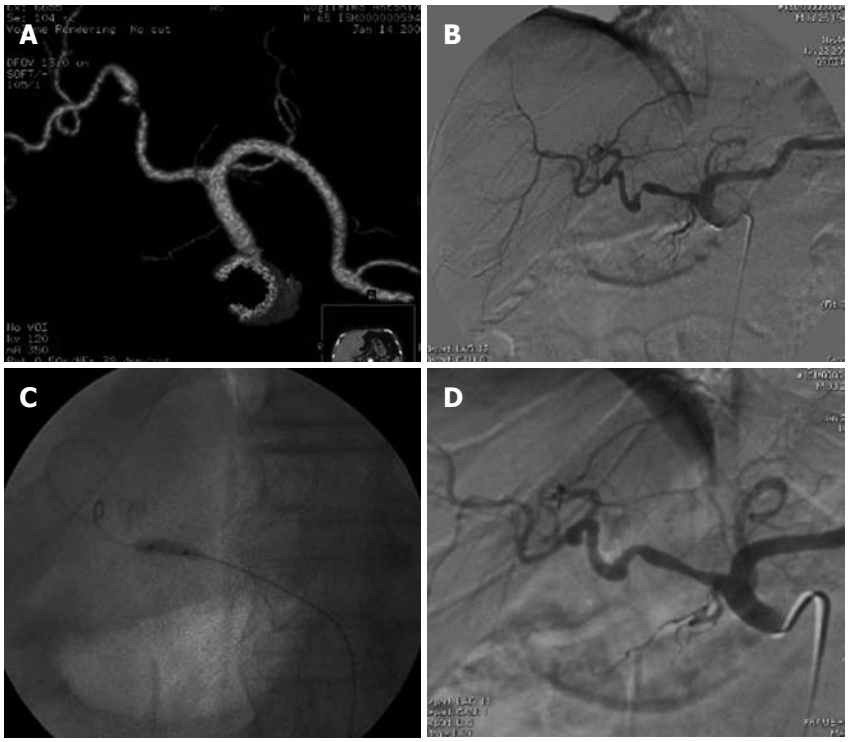

Figure 2 Status post deceased donor LT. Doppler US performed 5 mo after the transplant showed low intrahepatic resistive index 0.50 and prolonged systolic acceleration time 0.113 s, suspected for HAS. A: MDCT volume rendering 3D reconstruction showed a severe stenosis in the hepatic artery anastomosis; B: Digital subtraction angiography (DSA) celiac arteriogram confirmed the stenosis, trans-stenotic pressure gradient measured by a microcatheter was 50 mmHg; C: DSA percutaneous transluminal angioplasty performed by 4 mm diameter balloon catheter; D: DSA final arteriogram showed good patency of the arterial anastomosis with trans-stenotic pressure gradient reduced to 4 mmHg. Doppler US performed 4 mo later showed regular resistive index 0.70 and systolic acceleration time 0.100 s. Patient currently in good general condition and without biliary tree impairment after 6 mo of follow-up.

flow and uncovered underlying arterial anatomical defects in four out of five patients treated, but none were treated definitively by endoluminal procedures, due to the inability to resolve the underlying arterial stenosis, showing that the treatment of the underlying arterial defect is mandatory. Several cases of successful thrombolytic treatment of HAT in adult transplanted recipients are reported, usually if the diagnosis of HAT is performed a few hours after LT[12-15], but further analysis is needed to understand the correct timing of a possible thrombolysis and if endovascular procedures can be safely considered, a viable option to prevent re-transplantation after HAT. Possible complications of endovascular therapies are dissection and/or rupture of the hepatic artery during the arterial manipulation (requiring the placement of a covered stent) and bleeding from the arterial anastomosis[16].

Hepatic artery stenosis (HAS)

HAS is an insidious complication of LT occurring in approximately 5% of patients[17] leading to graft ischemia and possible hepatic artery occlusion as a result of slow flow with high incidence of morbidity and mortality due to hepatic insufficiency, biliary damage and possible sepsis. The majority of HAS arises at the anastomosis site and usually occurs within 3 mo after transplantation. The etiology of HAS can be due to small caliber of arteries or vascular clamp injury. Non-anastomotic stenosis can be present in cases of rejection or hepatic necrosis. The most common complication seen on cholangiography of recipients with HAS is non-anastomotic biliary strictures (BSs) seen in up to 49% of patients[18]. Early detection of HAS is fundamental because many of the stenoses are suitable for percutaneous treatment with angioplasty and/or stent placement or surgical revision, allowing good long-term graft function. In adult recipients with HAS that underwent percutaneous transluminal angioplasty, a 60%-80% patency rate at 1 year is reported[19]. Percutaneous transluminal angioplasty has also been reported to be an effective treatment of HAS after living donor LT, with a success rate of 94% and a complication rate of 6%, with possible HAS recurrence in 33% of patients[20]. In our practice, in a case of suspicious Doppler US or MDCT scan of HAS, a hepatic arteriogram is performed, from a transfemoral approach, with a 5F Cobra 2 or SOS catheter. A coaxial microcatheter is then advanced through the stenosis and the trans-stenotic pressure gradient measured. If a significant pressure gradient is present (> 10 mmHg) then an angioplasty is performed. Before angioplasty, 0.2 mg of nitroglycerine and 2000 UI of heparin are infused into the hepatic artery to reduce the risk of spasm or thrombosis. A 6F guiding catheter is advanced and a balloon catheter advanced over a 0.018 inch or 0.014 inch stiff wire. The diameter of the balloon used varies according to the diameter of the hepatic artery, ranging from 3 to 6 mm. Procedural success is determined by reduction or absence of the stenosis in a final arteriogram with significant reduction of the trans-stenotic pressure gradient (Figure 2). If a good patency is not restored, a metallic stent is deployed. The use of low-profile coronary stents in the treatment of HAS, as a first therapeutic approach, also showed good results with a 1-year patency rate of 45%-53%[21,22].

Portal vein stenosis (PVS)

PVS is a postoperative complication reported in 3% of patients after LT[23]. Clinical symptoms of hemodynamically significant PVS are related to portal

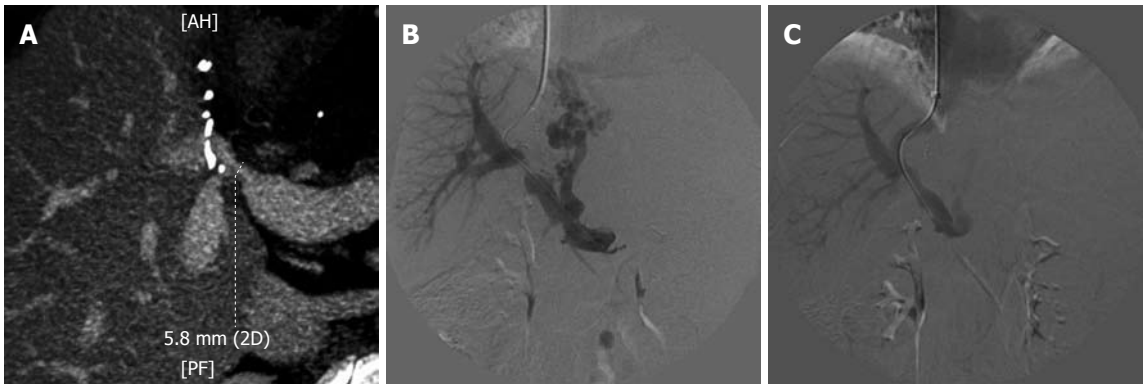

Figure 3 Status post right lobe LRLT in 60-year-old female. A: MDCT, MIP reconstruction showed a stenosis in portal vein anastomosis. The stenosis is very near the bifurcation of anterior and posterior branches; B: DSA, portogram performed from transjugular approach confirmed the PVS, 15 mmHg trans-stenotic pressure gradient was measured; note large patent coronary vein with filling gastro-oesophageal varices; C: DSA, final portogram performed after the deployement of a 10-mm diameter WallStent; note good filling of intrahepatic branches and no more evidence of the coronary vein, trans-stenotic pressure gradient reduced to 6 mmHg. Patient currently in good general condition with 1 year of follow-up.

hypertension and are bleeding from varices, splenomegaly and ascites. The portal vein is usually accessed by a transhepatic approach or by a transjugular approach. Percutaneous transhepatic angioplasty is considered an effective treatment and is usually considered as a first, non-surgical, therapeutic approach. Shibata *et al*[24] in a large series of patients, reported a success rate of 74% with a single session of balloon dilatation and a mean follow up of 24 mo. Recurrent stenoses were detected in 28% of patients and a maximum of three sessions of dilatation were necessary to resolve the stenosis. Funaki *et al*[25] reported metallic stent placement to treat recurrent and/or non-responsive, elastic stenosis with good long-term patency. Ko *et al*[26] reported a series of patients following living donor LT with early occurrence of PVS that were treated with transhepatic primary stent placement and showed good patency of the stents after a mean follow up of 66 mo in six out of nine patients. In the same paper, three post-procedural major complications were reported, two cases of hemoperitoneum and one case of intrahepatic pseudoaneurysm. In our practice, when a transhepatic approach is preferred, the procedure is performed under monitored anesthesia care. Transhepatic puncture of the portal vein is performed under ultrasound guide with a 21-Ga needle. An Accustik system (Boston Scientific) is advanced in the portal branch, over a nitinol wire, and then exchanged, over a 0.035 inch wire, for a 6F or 7F vascular sheath. The hemodynamic trans-stenotic pressure gradient measurement is performed using a 5F hydrophilic catheter. Before the dilatation, a bolus of heparin is administered (2000 UI) to reduce the risk of thrombosis during the balloon occlusion. The dilatation can be performed with balloon catheters up to 10 mm in diameter or more, according to the size of the vessel. Technical success is considered the resolution of the stenosis in a final portogram and a significant reduction of the trans-stenotic pressure gradient. In our practice, we embolize the transhepatic tracks with a coil to reduce the risk of bleeding, but in other series[25], transhepatic track embolization is not performed routinely without evidence of perihepatic bleeding. In patients with severe coagulopathy and/or ascites, the transjugular approach can be chosen, reducing the risk of bleeding[27,28]. The procedure is performed with monitored anesthesia care from the right internal jugular vein approach using the standard Colapinto set. Balloon dilatation and/or metallic stent placement are performed with the same technique as the transhepatic approach. Note that when the stenosis is very near the intrahepatic branches bifurcation, it is mandatory to use a non-covered metallic stent because the use of a covered stent, such as a Viatorr, would cause the occlusion of one branch (Figure 3).

IVC stenosis (IVCS)

IVC anastomosis stenosis after orthotopic LT is a rare complication occurring in approximately 1% of patients but more frequently in the superior anastomosis of IVC[29,30]. Clinical manifestations are usually refractory ascites and/or pleural effusion associated with renal insufficiency, lower extremities edema and alterations of liver function tests. IVCs is usually related to technical problems during surgery or fibrous scar development at the anastomosis, and concomitant stenosis at hepatic vein anastomosis can be present. For this reason, hepatic vein catheterization is recommended during the same procedure. Donor-recipient size mismatch can also be responsible for IVCS. Transluminal angioplasty is the first-choice treatment for this complication[31] (Figure 4). Trans-stenotic metallic stent deployment is reserved for resistant stenoses or those with elastic recoil not responsive to angioplasty[32]. A transfemoral approach is preferred for a diagnostic cavogram, as is trans-stenotic pressure gradient measurement. Filling of collateral peri-caval vessels is possible in case of hemodynamically significant stenosis. Balloon dilatation is performed with large-sized catheters. Due to the large size of the IVC, simultaneous inflation of multiple balloons has been described[1]. In our practice, during the procedure, radial or femoral artery pressure measurement is performed to continuously monitor changes in systemic hemodynamics during balloon dilatation, and consequent

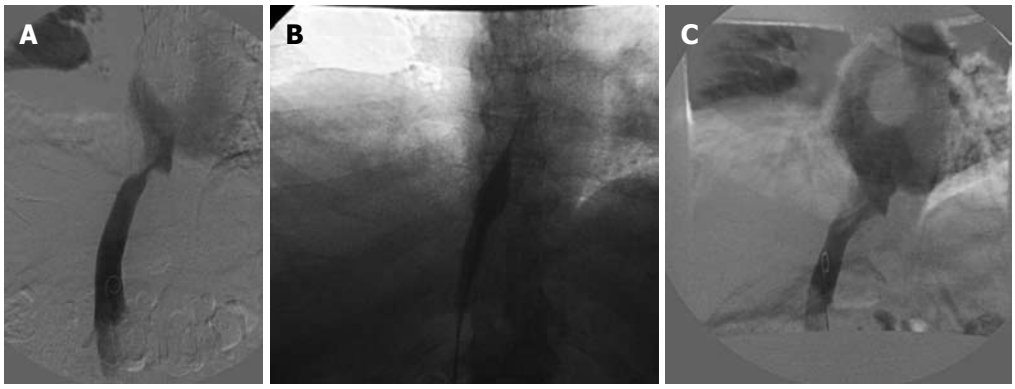

Figure 4 Status post deceased donor LT. Patient with refractory ascites in association with renal insufficiency and lower extremities edema 4 years after the transplant. A: DSA, cavogram showed a stenosis of IVC upper anastomosis, 12 mmHg trans-stenotic pressure gradient was measured. In the same session hepatic veins catheterization was performed showing no concomitant stenoses in hepatic vein anastomosis; B: DSA percutaneous transluminal angioplasty performed by 16-mm diameter balloon catheter; C: DSA final cavogram showed good patency of the caval anastomosis with trans-stenotic pressure gradient reduced to 2 mmHg. From 2001 to 2007, four other trans-luminal caval angioplasties were performed in the same patient. The patient is currently in good general condition after 15 mo and has avoided re-transplantation.

IVC occlusion, deflating the balloon before an excessive drop of systemic pressure. Repeat dilatations may be necessary for long-term patency. In patients with IVCS recurrence and severe renal insufficiency, trans-anastomotic pressure gradient measurement and balloon dilatation can be easily performed without injection of iodinate contrast.

Hepatic vein stenosis (HVS)

HVS, inducing outflow insufficiency, is a major postoperative complication of LT, especially in patients with partial liver graft transplantation producing graft failure with a reported incidence of 1%-4%[33-35]. Hepatic congestion can cause refractory ascites, refractory hydrothorax and alteration of liver function tests. HVS usually occurs at the anastomosis site; less frequent is the presence of an intrahepatic stenosis in the HV, likely due to previous venous injury during surgery or during previous percutaneous procedures, such as biopsy or biliary catheter placement. If a clinical and/or imaging suspicion of HVS is present, selective catheterization of all the HVs is mandatory to confirm the stenosis and measure the trans-stenotic pressure gradient (Figure 5). A pressure gradient greater than 3 mmHg between the HV and right atrium has been reported to be pathological[35]. Transjugular or transfemoral angioplasty or metallic stent placement is usually performed, as a first choice, to treat this complication[31,33-35]. In our experience, balloon dilatation is considered the preferred treatment choice because long-term patency of metallic stents is still unknown and metallic stent placement is reserved for persistent HVS not responsive to multiple angioplasties. Good technical and clinical success rates for percutaneous interventions are reported[33-36]. Long-term patency may require repeated interventions, especially if only trans-luminal angioplasty is performed. Better long-term patency results are reported in cases of stent deployment[36]. The primary percutaneous transhepatic approach for HVS treatment, has been reported[35] to have an easier negotiation through the stenosis, leading to shorter procedure time and ionizing radiation. In our experience, we use the percutaneous transhepatic approach only when the transjugular or the transfemoral approach fails. For the transhepatic approach, previous drainage of ascites and the embolization of the transhepatic tracks at the end of the procedure are, in our opinion, mandatory to reduce the risk of bleeding.

TREATMENT OF BILIARY COMPLICATIONS

Biliary complications occur in 10%-40% of patients after LT[37-40], with major incidence in patients with partial LT[41-42]. Complications include BSs, bile leakage (BL), biliary stones and bilomas. The majority of biliary complications develop during the first 3 mo, but strictures and stones may develop months or years after LT. The preferred methods for biliary tract reconstruction in LT are the duct-to-duct anastomosis between the donor and recipient common ducts and, less frequently, Roux-en-Y choledocojejunostomy. In right lobe split LT (from deceased or living related donor), a duct-to-duct anastomosis is usually performed between the donor right biliary duct and recipient bile duct, but due to possible anatomical variants of the donor biliary tree, two different biliary anastomoses, or less frequently three anastomoses, are performed with the recipient bile duct and the recipient cystic duct or with a Roux-en-Y limb, in up to 40% of patients[41,42]. Patients with multiple biliary reconstructions have a higher incidence of biliary complications[42]. Clinical presentation of biliary complications is often indistinguishable from other post-transplantation complications (vascular complications, rejection, graft dysfunction, infections). Ultrasonography is commonly used as screening test; however, due to the high rate of false-negative results, a negative test cannot exclude the presence of biliary complications.

Endoscopic retrograde cholangiopancreatography (ERCP) is usually the initial method of choice to

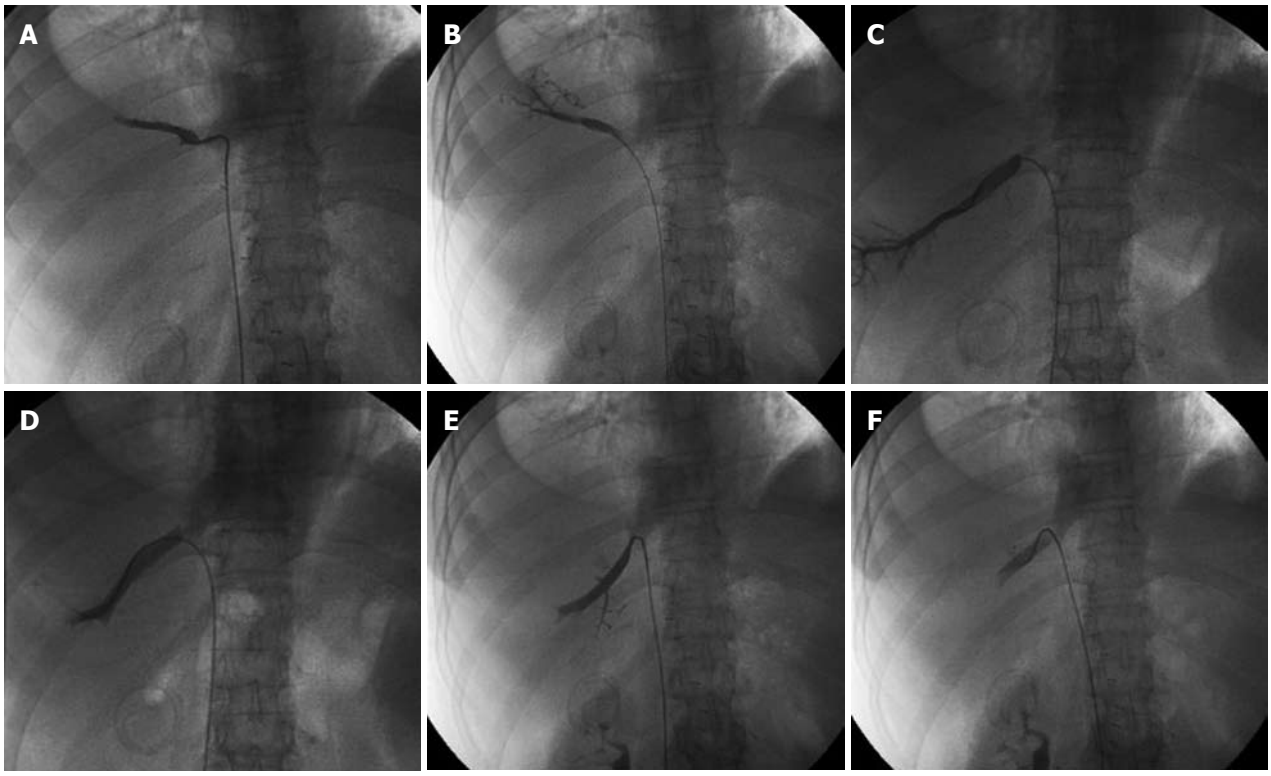

Figure 5 Status post deceased donor right lobe split LT in 65-year-old female. Three separates HV anastomoses were performed. One month after LT, patient developed refractory ascites and right pleural effusion with worsening of liver function tests. Doppler US was suspicious of HVs. A cavogram performed from the femoral approach showed widely patent IVC anastomosis. Selective catheterization of the three HVs (A, C and E) showed stenosis in the three anastomoses with trans-stenotic pressure gradient of 15, 20 and 16 mmHg, respectively. Balloon angioplasties were performed with balloon catheters ranging from 7 to 10 mm in diameter. Final venogram showed patent anastomoses (B, D and F). Trans-anastomotic pressure gradient reduced to 4, 2.5 and 8 mmHg. Eight months of follow-up without recurrence of refractory ascites and/or hydrothorax.

treat post LT biliary complications in patients with duct-to-duct anastomosis. Percutaneous transhepatic cholangiography (PTC) is the method to manage biliary complications in patients with choledochojejunostomy, in presence of intraepatic strictures and when endoscopic management fails.

Biliary strictures (BS)

BS is a common problem after LT with a reported incidence of 10%-35%[37-40]. Anastomotic BS is usually related to scar tissue and retraction at the suture site [41-42]. Intrahepatic BS is usually related to chronic rejection or arterial insufficiency due to HAS, thrombosis or ABO blood group incompatibility or infections. Single focal strictures and multiple/combined intrahepatic and anastomotic strictures can be present. Untreated BS is associated with high rate of morbidity and mortality. Endoscopic intervention is the preferred approach in patients with duct-to-duct anastomosis. PTC with biliary drainage placement and consequent percutaneous balloon dilatation is performed in patients with the Roux-en-Y reconstruction or in cases of endoscopic failure. In patients with partial LT, knowledge of the number of the anastomoses performed is mandatory before a possible percutaneous treatment. Possible complications of PTC are hemobilia, drop in hematocrit, intra or extrahepatic hematoma, fever with bacteremia, with a reported incidence of 3%-26% of cases[43,44]. Severe injury to intrahepatic arteries with massive hemobilia and possible formation of intrahepatic aneurysm is reported in 2% of cases. In those cases, emergency arterial embolization is required[43]. Suspicion of BS is based on one or more findings: clinical picture (fever, cholangitis), biochemistry (elevation of alkaline phosphatase, direct bilirubin, and transaminases), ultrasound and/or CT scan and/or MR (biliary duct dilatation), and liver biopsy (with histology consistent for cholestasis due to biliary obstruction). BS can also be present in cases of non-dilated biliary ducts. An ultrasound sensitivity of 38% in the detection of biliary obstruction has been reported in transplanted patients[45]. Better results, with sensitivity in detecting biliary obstructions ranging from 80% to 100%, are reported with the use of magnetic resonance cholangiography (MRCP)[46]. Percutaneous treatment of BS is considered safe and effective, avoiding in most cases the need for surgical revision of the anastomosis. Multiple treatments are often necessary. Long-term patency of percutaneous bilioplasty in adult recipients is reported from 50% to 60% at 5 years[47,48]. Prolonged cold ischemic and operative times, multiple or peripheral strictures and the presence of hepatic artery disease, predispose to treatment failure or a lower patency of anastomotic BS after balloon dilation[48,49]. The use of cutting balloon catheters or combined cutting and conventional balloon protocol has been proposed in patients with refractory anastomotic stricture[50,51].

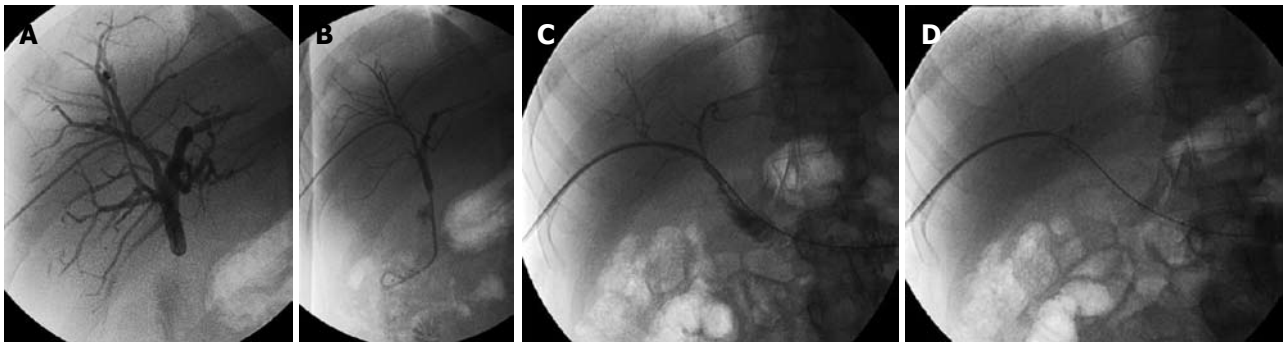

Figure 6 Status post deceased donor LT in 40-year-old man. Hepatico-jejunostomy was performed. A: PTC shows moderate biliary duct dilatation and subocclusive biliary anastomosis stricture; B: The stricture was crossed and trans-anastomotic. 6F Ring biliary catheter was placed; C: cholangiogram performed after four sessions of percutaneous bilioplasty shows no bile duct dilatation and patent anastomosis; D: Evidence of complete contrast transition from the bile ducts into the bowel loop within 2 min after the cholangiography. Three years of follow-up without clinical evidence of stricture recurrency.

Metallic stent deployment is reserved for patients who are refractory to repetitive balloon dilation of BS and who are poor surgical candidates[52]. In our practice, PTC is performed in an angiographic suite under monitored anesthesia care, with spontaneous respiration and additional local anesthesia. The patient is monitored continuously by electrocardiography, a pulse oximeter, and automatic blood pressure and pulse recordings. Intravenous antibiotic prophylaxis is administered before all procedures. Patients with coagulation defects (platelets < 50 000 mm^3 and/or prothrombin activity < 50%) receive infusions of platelets and/or fresh frozen plasma. PTC is generally performed through an intercostal approach, with an ultrasound- and fluoroscopy-guided 20-Ga needle inserted in a peripheral bile duct. If the cholangiography shows a stricture, the biliary tree is catheterized using an Accustick Introducer System (Boston Scientific, Natik, USA) over a Cope wire (Cook, Bjaeverskov, Denmark), the stricture crossed when possible with 0.035 or 0.038 inch hydrophilic guide wires, and a trans-anastomotic biliary catheter ranging from 6F to 8.5F with side holes placed above and below the stricture. The catheter is left to external gravity drainage for at least 1 d. If the patient has no fever and/or cholangitis the day after the procedure, the catheter is clamped for internal drainage. If, after the diagnostic cholangiogram, a guide wire cannot be passed through the stricture, an external drainage catheter is placed to allow for biliary decompression and to reduce the stricture's possible inflammatory component. A second attempt to cross the stricture is usually performed after 7 d. The first BS balloon dilatation session is never performed on the same day as the diagnostic cholangiogram, so as to reduce the risk of sepsis. It is generally performed after 1 wk, following a cholangiography performed with a 6F or 7F sheath and a balloon size ranging from 6 to 10 mm. Every dilatation session consists of three trans-anastomotic dilatations of 10 min each. Trans-anastomotic biliary catheters with sizes ranging from 6F to 14F according to the diameter of the anastomosis are placed after every dilatation session. An antibiotic infusion is re-administered 6 h after every procedure. At each dilatation session, the size of the balloon catheter is increased by 1 mm, up to a maximum diameter of 12 mm. Catheters are removed when the cholangiography performed through the sheath shows evidence of stricture resolution or of minimal residual stenosis of less than 20% of the expected lumen caliber. In all cases, catheters are removed only upon evidence of complete contrast transition from the bile ducts into the bowel loop within 3 min after the cholangiography (Figure 6), and if a significant reduction of cholestasis serum liver enzymes is achieved after bilioplasty. Our protocol envisages three BS dilatation sessions performed every 4-8 wk, followed by a cholangiographic evaluation 4 wk after the last session. If the stricture is resolved, the catheter is removed; if the BS persists, a supplementary balloon dilatation and follow up cholangiogram is performed, followed by potential catheter removal or balloon dilatation after 4-6 wk. In our experience, when a BS is crossed and a biliary catheter placed, percutaneous balloon dilatation gives good results, although multiple sessions over several months are necessary to obtain stricture resolution.

Bile leakage (BL)

Post-operative BL is a complication of LT that usually occurs within a few weeks from the transplant in 5%-20% of patients[37-39,53]. BL can arise from bile duct anastomosis of the resection margin in partial LT. Another possible site of leakage is the T-tube insertion in patients with choledocho-choledocho anastomosis. Small leakages usually close spontaneously, while large BLs are a serious complication and need to be treated because of possible associated complications such as fever, abdominal pain, fluid electrolyte depletion, fat malabsorption, possible sepsis or bleeding for hilar vascular erosion. The initial management should be non-operative. Percutaneous drainages, placed with a sonographic guide, are used to drain the bile collection. Endoscopic or percutaneous transhepatic management, with placement of large-size biliary catheters, allows achievement of good results in the treatment of large BL in adult patients, avoiding surgical repair in many cases[54-58]. In selected patients, when prior endoscopic or percutaneous transhepatic attempts to stent

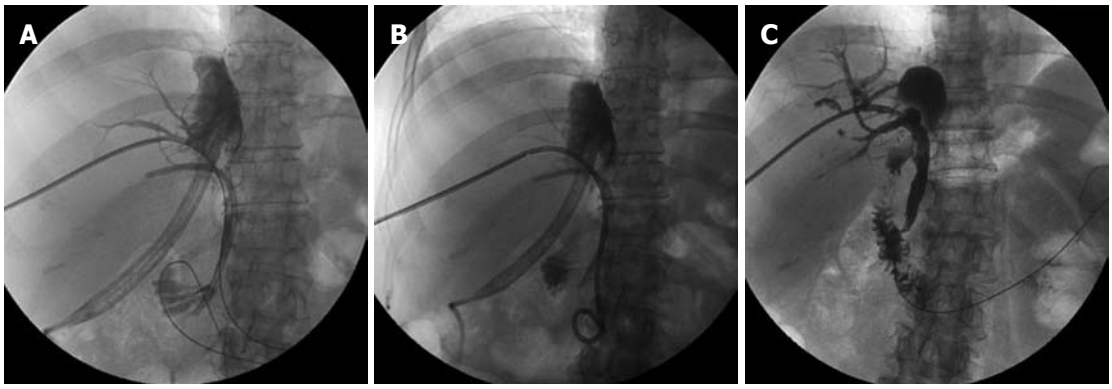

Figure 7 Status post LRLT (right lobe) in 56-year-old woman. Two separate biliary anastomoses were performed. Approximately 300 mL of bile was drained every day from the existing perihepatic drainage catheter (JP) placed during the transplantation. ERCP was performed, revealing a BL from the anastomotic region. An endoscopic stent was deployed in the posterior duct anastomosis but endoscopy failed to place a stent in the anterior duct anastomosis. A: PTC of the anterior duct showed no bile ducts dilatation and a BL arising from the anastomosis; B: 12F external internal catheter without side holes was placed in the leak region. The bile output from the JP progressively reduced and stopped a few days later. The JP and the endoscopic stent were removed 1 mo later; C: Final cholangiogram performed 4 mo later showed no BL and patent biliary anastomosis. The catheter was removed. The patient is still in good condition after 12 mo of follow-up.

the biliary tree have failed, the combined transhepatic-endoscopic approach (rendezvous technique) can be successfully used to place large-size biliary catheters[59-60]. When minimally invasive treatments fail, operative intervention is mandatory. In our practice, in patients with anastomotic BL who undergo percutaneous trans-hepatic treatment, we do not use biliary catheters with standard side holes, but we prefer to modify multipurpose large-size drainages by adding holes only in the intrahepatic bile ducts and in the distal bile duct, so as to reduce bile contact in the duct lesion to favor the repair process (Figure 7).

CONCLUSION

LT, in patients with end-stage liver diseases, has become an accepted treatment. Advances in the field of percutaneous, radiological, minimally invasive techniques have increased the importance of interventional radiology in the management of patients after LT. Interventional radiology procedures are used in the treatment of vascular and non-vascular complications, improving graft and patient survival and avoiding, in the majority of cases, surgical revision and/or re-transplantation.

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TOPIC HIGHLIGHT

Salvatore Gruttadauria, MD, Associate Professor, *Series Editor*

Psychological evaluation and follow-up in liver transplantation

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Abstract

An increasingly number of transplant centers have integrated a psychological assessment within their protocol for evaluation of patients being considered for transplantation. This paper highlights the psychological criteria for inclusion or exclusion for listing, briefly discusses the psychological dynamics of patients, and addresses possible psychotherapy and pharmacological therapy, before and after transplant.

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Key words: Liver transplantation; Psychology; Contraindications; Cognitive behavior therapy

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INTRODUCTION

Orthotopic liver transplantation (OLTx) is a major surgical procedure that can precipitate distress, anxiety and depression. The experience of the last few years of many transplantation centers has highlighted the importance of a thorough and routine psychological assessment before considering the patient as a possible candidate for listing[1]. The importance of identifying psychological/psychiatric, and/or possible psychosocial problems is necessary in order to eliminate or prevent the insurgence of possible psychological problems post-transplant. Most transplant centers have included an initial psychological evaluation in their work-up protocol, to evaluate the psychological strengths and possible liabilities of the patient who is being considered for an OLTx, so as to provide interventions such as: smoking cessation therapy, drug/alcohol rehabilitation, and improvement of compliance; that is, behavior that needs to be resolved before surgery, in order to reduce possible behavioral liabilities after transplantation[2].

The transplant itself has deep psychological implications, which may exist within the affective, social and interpersonal realm of the individual's personality. In the postoperative phase, there may be manifestations of adjustment disorders, psychopathological disturbances, problems with compliance, as well as non-adherence to the therapeutic plan[3]. To reiterate, it is therefore necessary to carry out an accurate evaluation of the psychological and personality profile of each individual being considered for listing for possible OLTx.

PSYCHOLOGICAL ASSESSMENT OF THE POTENTIAL CANDIDATE FOR TRANSPLANT

During the initial interview, the psychologist's main goal is to determine how much the candidate knows or is aware of his/her medical status, or better yet, whether he/she has accepted his/her medical condition. The communication of the necessity of a liver transplant automatically induces the patient to think that conventional therapies and/or less invasive surgery are no longer an option. In such a case, the patients' psychological-emotional reactions take a course of their own, for example, they start experiencing: (1) sense of despair; (2) concerns for his/her medical status and sense of imminent death; (3) reactive and/or correlated psychopathology.

During the course of the psychological evaluation, then, patients may find themsleves living two traumatic events (both real), at the same time: (1) sense of imminent death; or (2) rebirth through the transplant. During this phase, it has been observed that patients most often feel a sense of doubt, anxiety, ambivalence, fear and frustration,

which, if associated with a high level of psychological distress, can have consequences that can even lead to non-acceptance of the transplant. A careful psychological evaluation (cognitive, emotional and interpersonal) allows for an accurate course of psychotherapy[4].

The therapist must take into consideration those needs, deficits and assets that the patients possess in order to bring them step by step toward the final objective, which is the transplant. The specific, individualized treatment plan allows for an improvement of the quality of life (QOL) of the patient who will undergo a transplant and, specifically, during the postoperative period[4].

Ethically, the ability to give informed consent comprises three key elements: adequate information, adequate decision-making capacity, and freedom from coercion (President's Commission for the Study of Ethical Problems in Medicine and Biomedical and Behavioral Research, 1982)[5]. Therefore, to this end, it appears fundamental to the psychologist's evaluation, whose goal is to evaluate the degree of the patient's knowledge and understanding of the various items on the informed consent. To this extent, it is fundamental that the patient's ability for decision-making is evaluated. If there is a suspected inability to do this (because of mental retardation or social deficits, *etc*), then, it is imperative that further testing be done using standardized tools for the evaluation of IQ (e.g. Wechsler Adult Intelligence Scale-Revision). If mental deficiency is found, then it becomes a legal issue, that is, it is necessary to assign a legal guardian who can represent the patient, in order to protect his/her rights. In the absence of a cognitive defect, if it is evaluated that the patient has not fully understood the context of such a document because of difficulty in perceiving such information, or in the event that there is resistance in accepting such information, then it becomes imperative that the patients undergo a psycho-educational process, in order to induce them to adjust their non-functional behavior or lifestyle in accordance with the expectations of the transplant team. For example, patients might need to be educated on topics such as maintaining adequate personal hygiene, given that they will be treated with immunosuppressant medication for the rest of their lives.

Table 1 outlines the absolute and relative contraindications for transplant listing. Although each item needs detailed discussion, for the purpose of this paper, the discussion will focus on alcohol/drug addiction and psychopathology, which are the two contraindications that need the most active intervention of the psychologist. The candidacy of patients who have an addiction has varied within each transplant center; however, in recent years, there has been an attempt to formalize the criteria for such patients. In Italy, the Director of the National Transplant Institute assigned a group of psychologists and psychiatrists to work on the guidelines to be applied in transplant centers across the country. This group (GLI PSI TO), of which the present author is a member, debated and focused a lot of time and energy in determining the criteria for listing patients with addiction. The consensus was, also following the lead of guidelines set forth by the United Network for Organ Sharing, that patients may be considered for listing after 6-12 mo abstinence, and that they have to be active participants in a rehabilitation center (even as an out-patient). With such patients, during this period of abstinence at our center (Istituto Mediterraneo per Trapianti e Terapie ad alta Specializzazione; ISMETT), the treatment is two-fold: patients are sent to a rehabilitation center closest to their residence, where the main focus is the toxicological component of the problem; while at ISMETT, the psychologists work in full synergy with such centers in an attempt to give patient support, in order to access those possible psychosocial resources that are needed for a positive, favorable prognosis.

Table 1 Contraindications for OLTx

Absolute
Irriversible cognitive-neurological deficits
Active psychosis
Active addiction to drugs and/or alcohol
Relative
Personality disorder
History of psychiatric disorders
History of alcohol and/or drug addiction
Depression
Neurosis
History of use of psychotropic/neuroleptics
Limited family and social support
Limited ability to adhere to therapies
Inadequate motivation

With regard to psychopathology, it is important to note that it is not always a contraindication for transplantation *per se*. In fact, if patients manifest an active psychosis, not well compensated even with pharmacological therapy[6], it is obvious that this would be an absolute contraindication, especially since there is an absence of the necessary resources needed to undergo an OLTx. In other cases, however, such as in mood disorders and anxiety disorder, psychopharmacological therapy in conjunction with psychotherapy may ameliorate the disturbance to the point at which patients are placed in a condition in which they can reach a functional emotional, affective equilibrium that allows them to manage the eventual distress related to the transplant. Such patients, however, need constant support before, during and after transplantation. During the pre-transplantation phase, specifically for sensitivity to stress; in the post-transplant phase, most importantly because of immunosuppressant therapy that might precipitate mood swings, irritability, mania and anxiety. Psychotherapy and/or psychotherapy in conjunction with pharmacological treatment might be indicated during all the phases of the transplant process. Cognitive behavior therapy is the psychotherapeutic approach implemented at ISMETT, an approach which has been evaluated as being most beneficial with these patients, as they are individuals who tend to manifest traits such as depression, anxiety and phobia. Anxiety reduction techniques, autogenic training, systematic desensitization,

Table 2 Domains of the pre-transplant psychological evaluation

Informed consent
Personality profile
Psychopathology
Past/present psychiatric history
Effect of illness on daily life activities
Defense mechanism employed and coping skills
Motivation for surgery
Treatment compliance
Support from the family
Socioeconomic support (together with social worker's evaluation)
Awareness of information regarding the actual surgical event and future treatments
Use/abuse of alcohol and/or drugs (see paragraph on this topic)
QOL

relaxation techniques, guided imagery, pain management and hypnosis are techniques that might be implemented, and that normally bring more immediate results for the management of those symptoms already mentioned as those being manifested by patients during the transplant process (Table 2).

CONCLUSION

The role of the psychological assessment and monitoring during the pre- and post-transplant phases, as well as the ongoing follow-up intervention, is generally highly valued by organ transplant teams because of the significant health consequences of organ transplant failure. Identifying and reducing psychological risk factors can play an important role in overall long-term success of transplantation.

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N-cadherin knock-down decreases invasiveness of esophageal squamous cell carcinoma *in vitro*

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Abstract

AIM: To examine the expressions of N-cadherin and E-cadherin in specimens of 62 normal esophageal epithela, 31 adjacent atypical hyperplastic epithelia and 62 esophageal squamous cell carcinomas (ESCCs), and to investigate the roles of N-cadherin in the invasiveness of ESCC cell line EC9706 transfected by N-cadherin shRNA.

METHODS: PV immunohistochemistry was used to detect the expression pattern of N-cadherin and E-cadherin in specimens of 62 normal esophageal epithelia, 31 adjacent atypical hyperplastic epithelia and 62 ESCCs. The invasiveness of ESCC line EC9706 was determined by transwell assay after EC9706 was transfected by N-cadherin shRNA.

RESULTS: The positive rates of N-cadherin decreased in the carcinoma, adjacent atypical hyperplastic and normal esophageal tissues (75.8%, 61.3% and 29.0%, $P < 0.05$), respectively, while those of E-cadherin increased (40.3%, 71.0% and 95.2%, $P < 0.05$). The increased expression of N-cadherin and decreased expression of E-cadherin were related to invasion, differentiation, and lymph node metastasis ($P < 0.05$). The expression level of N-cadherin decreased in the N-cadherin knocked down cells, and the invasiveness of those cells decreased significantly as well. The number of cells which crossed the basement membrane filter decreased from 123.40 ± 8.23 to 49.60 ± 6.80 ($P < 0.05$).

CONCLUSION: E-cadherin and N-cadherin expression is correlated with the invasion and aggravation of ESCC. The down-regulation of N-cadherin lowers the invasiveness of EC9706 cell line.

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Key words: Esophageal squamous cell carcinoma; RNAi; N-cadherin; EC9706

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INTRODUCTION

The malignancy of cancer cells depends largely on their proliferative, invasive and metastatic activities, and invasive and metastatic activities are most closely associated with cell-to-cell and cell-to-extracellular matrix adhesion. As a class of transmembrane proteins, cadherins play an important role in cell adhesion. Among the members of cadherin family, E-cadherin and N-cadherin have been extensively studied about their biological activities and associations with cancer cell invasion. It was reported that the invasion and metastasis were present in the esophageal squamous cell carcinoma (ESCC) with a low level of E-cadherin[1,2]. Recent studies on prostate cancer and breast cancer proved that the up-regulated N-cadherin plays an important role in cell progression and metastasis[3,4]. In addition, N-cadherin is involved in angiogenesis and tumor growth regulation, and contributes to the invasive morphology in squamous tumor cells, and stimulates migration, invasion and metastasis[5]. However, the association of E-cadherin and N-cadherin expression with the malignancy of the ESCC is unknown.

In the current study, we analyzed the expression of E-cadherin and N-cadherin in the ESCC tissues, adjacent atypical hyperplastic epithelium and normal esophageal

epithelium. The roles of N-cadherin in the invasiveness were investigated in ESCC cell line (EC9706) transfected by N-cadherin shRNA.

MATERIALS AND METHODS

Tissue samples

In this study, we randomly selected 62 esophageal cancer patients who underwent potentially curative surgery without preoperative chemotherapy or radiotherapy between February 26 and March 16, 2006 in Anyang Tumor Hospital, Henan, China. Among them, 36 were men and 26 were women, ranging from 38-75 years of age with a mean age of 52.6 years. Overall, 22 cases were poorly differentiated, 25 were moderately differentiated, and 15 were well differentiated. For the lymphatic node metastasis, 20 cases were positive and 42 cases were negative. Seven cases were classified as T_1 and T_2, and 55 as T_3 and T_4 in terms of T stages. Surgically removed specimens were routinely fixed in buffered formalin and embedded in paraffin blocks for clinical diagnosis and reclassification for this study. The normal esophageal squamous tissues were taken from mucosae 3 cm away from carcinomas, and the adjacent atypical hyperplastic epithelium 2 cm away from the carcinomas. The final pathological diagnosis was based on the result of histological examination.

Expression of E-cadherin and N-cadherin in ESCC

Immunohistochemical analysis for E-cadherin and N-cadherin was performed on 5-μm sections made from tissue microarray blocks. The Envision Plus detection system (Dako, Carpinteria, CA, USA) was used for the immunostaining. The sections were deparaffinized in xylene and then were microwaved in 10 mmol/L citrate buffer (pH 6.0) to unmask the epitopes. Endogenous peroxidase activity was blocked by incubation with 0.03% hydrogen peroxide in methanol for 5 min. Slides were incubated with mouse anti-human E-cadherin antibody (microwaving retrieval in citrate buffer at 1:100 concentration, Abcam) and mouse anti-human N-cadherin antibody (microwaving retrieval in low pH buffer at 1:100 concentration; Abcam), respectively. Then, polyperoxidase anti-mouse IgG (Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., China) was added for incubation for another 30 min followed by gentle rinsing with washing buffer for three times. Thereafter, the sections were stained for 5 min with 3,3-diaminobenzidine (DAB) (Beijing Zhongshan Golden Bridge Biotechnology), counterstained by hematoxylin, dehydrated, and mounted in Diatex. Expression of E-cadherin and N-cadherin in breast cancer was used as a positive control, while the same concentration of PBS was applied as a negative control for E-cadherin and N-cadherin.

Evaluation of staining for E-cadherin and N-cadherin

For both E-cadherin and N-cadherin, only membrane and cytoplasm staining was considered as positive. The intensity and percentage of immunostained carcinoma cells were all taken into consideration according to the previously published method with modification[6]. Briefly, the extent of positivity was scored as 0 when no positive cell was observed; 1 when the percentage of positive cells was < 30%; 2 when it was 30%-60%; and 3 when it was > 60%. The intensity was scored as 0 when no positive cells were identified; 1, weak; 2, moderate; and 3, strong staining. Multiplying the extent by intensity gave the following immunohistochemical staining grades as 0, 1, 2, 3, 4, 6 and 9. For statistical analyses, grades 0, 1 and 2 were considered as negatively stained, and grades > 2 were considered as positively stained.

Retroviral virus production and determination of viral titer

PT67 packaging cells were seeded onto a six-well plate at 1×10^5 cells per well and incubated for 24-48 h. The cells were allowed to grow to 60%-70% confluence and then rinsed with fresh DMEM medium. The control vector pEGFP-MSCVneo and recombinant retroviral vector pMSCVneo/N-cadherin plasmids (kindly provided by Dr. Ma Jie, Chinese Academy of Medical Sciences, China) (The former contained the enhanced green fluorescent protein and neomycin resistance genes, the latter contained the N-cadherin shRNA, U6-promotor, enhanced green fluorescent protein and neomycin resistance genes) were transfected into the packaging cell line PT67 by lipofectamine 2000 (Invitrogen Corp, UK) and incubated for 6 h following the manufacturer's instructions. The mixture was replaced with fresh medium to stop transfection and the transfected PT67 cells were further cultured with G418 (1000 mg/L) selecting medium for 2 wk. Viral supernatant of the drug-resistant clones was filtered through a 0.45-μm filter and condensed by ultracentrifugation at low temperature, then preserved at -80℃. The supernatant, from the fresh PT67 packaging cells containing recombinant retrovirus, was used to infect NIH3T3 cells to determine viral titer as previously described[7]. The highest titer clones were selected for further experiments.

Infection of EC9706 cells with viral supernatant

EC9706 cells were plated in a six-well plate at 5×10^4 cells/mL and cultured with 5% CO_2 at 37℃. Twenty-four hours later, the cells were exposed to 2 mL viral supernatant at a consecutive multiplicity of infection once for 12 h, in the presence of 8 μg/mL polybrene (Sigma). Subsequently, viral supernatant was replaced with fresh medium. After being incubated for another 24 h, the infected EC9706 cells were screened with G418 (600 mg/L) (Sigma) selecting medium for 2 wk. Drug-resistant clones were expanded with G418 (300 mg/L) (Sigma) selecting medium. Two days after culturing, the expression of EGFP in infected EC9706 cells was observed under fluorescence microscope.

Total RNA and protein isolation

Total RNA and protein isolation was performed using the Macherey-Nagel total RNA and protein isolation kit according to the user manual. About 5×10^6 EC9706

Table 1 Real-time PCR primers for N-cadherin, E-cadherin, MMP-9 and GAPDH

Target gene	Primers	Length of product (bp)
N-cadherin	Sense: 5'-GGTGGAGGAGAAGAAGACCAG-3' Antisense: 5'-GGCATCAGGCTCCACAGT-3'	72
E-cadherin	Sense: 5'-CCCGGGACAACGTTTATTAC-3' Antisense: 5'-GCTGGCTCAAGTCAAAGTCC-3'	72
MMP9	Sense: 5'-GAACCAATCTCACCGACAGG-3' Antisense: 5'-GCCACCCGAGTGTAACCATA-3'	67
GAPDH	Sense: 5'-AGCCACATCGCTCAGACA-3' Antisense: 5'-GCCCAATACGACCAAATCC-3'	66

cells transfected with N-cadherin RNAi, control vector, or the untreated cells were collected and lysed. Through the NucleoSpin RNA/Protein column, RNA and DNA were bound to the column and protein was contained in the flow-through. After digestion of DNA, total RNA was isolated by washing the column. Protein was isolated from the flow-through and incubated for 3 min at 98℃ for dissolving and denaturation, and stored at -20℃ until used. All of the preparation and handling steps of RNA took place in a laminar flow hood under RNase-free conditions.

cDNA synthesis

RNA quality and quantity were determined by absorbance readings at 260 and 280 nm with the Nano Drop (ND-1000) spectrophotometer. RNA integrity was tested by PCR amplification of the GAPDH gene. Reverse transcription of RNA was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche). cDNA was synthesized from 5 μg total RNA isolated from EC9706 cells transfected with N-cadherin siRNA, control vector, or the untreated cells according to the manufacturer's handbook.

Primer/probe design

The primer pairs and hydrolysis probes for N-cadherin, E-cadherin, matrix metalloproteinase-9 (MMP-9) and GAPDH were designed by Universal ProbeLibrary Assay Design Center (Roche). All primer sequences listed in Table 1 were synthesized by Shanghai Sangon (China). The hydrolysis probes were designed and synthesized by Roche Diagnostics.

Real-time PCR

Real-time PCR was performed with the ABI Prism 7500 Sequence Detection System (ABI) in a total volume of 20 μL in glasscapillaries containing 2 μL of cDNA, 0.5 μmol/L of each primer, 0.1 μmol/L of hydrolysis probe and 4 μL of LightCycler TaqMan Master Mix (Roche Diagnostics). PCR reaction was initiated with a 12-min denaturation at 95℃ and terminated with a 30-s cooling step at 40℃. The cycling protocol consisted of denaturation at 95℃ for 10 s, annealing at 54℃ for 10 s, and extension at 72℃ for 10 s, and was cycled 45 times. Fluorescence detection was performed at the end of each extension step. The housekeeping genes GAPDH and DEPC-H_2O were set as internal control and negative control, respectively.

Western blot analysis

Twenty microliters of protein samples were separated on a 10% SDS-acrylamide gel (Bio-Rad) for 1 h at 150 V, and the proteins were transferred to nitrocellulose membrane (Whatman). After blocking in 5% fat-free milk, the membrane was treated with the dilution of the primary antibody overnight at 4℃ and the dilution of the secondary IgG-horseradish peroxidase (HRP) conjugated antibody for 1 h at room temperature. All dilutions were in PBS containing 5% Blotto (Santa Cruz) and 0.1% Tween-20. The stained membranes were visualized by enhanced chemiluminescence reaction using the ECL Plus (GE Healthcare). Western blot experiments were repeated at least three times on every sample, with similar results.

Transwell chamber migration assay

Matrigel-coated filter inserts with 8-μm pores that fit into 24-well invasion chambers were obtained from Becton Dickinson. EC9706 cells transfected with N-cadherin RNAi, control vector, or the untreated cells were detached from the tissue culture plates, washed, resuspended in conditioned medium (10^6 cells/mL), and then added to the upper compartment of the invasion chamber with or without plasmin (1.8 mg). Conditioned medium (500 μL) was added to the lower compartment of the invasion chamber. The Matrigel invasion chambers were incubated at 37℃ for 24 h in 5% CO_2. After incubation, the filter inserts were removed from the wells, and the cells on the upper side of the filter were removed using cotton swabs. The filters were fixed, mounted, and stained according to the manufacturer's instructions. The cells that invaded through the Matrigel were counted on the underside of the filter. Three to five invasion chambers were used for each experimental condition. The values obtained were calculated by averaging the total number of cells from three filters.

Statistical analysis

χ^2 test and Spearman rank correlation coefficient analysis were used to assess the univariate association between the immunohistochemical status and the clinicopathological characteristics. Results were expressed as mean ± SD. Statistical analysis was made using one way ANOVA or paired-samples *t* test of SPSS 11.0. $P < 0.05$ was considered statistically significant.

RESULTS

Immunoreactivity and clinicopathological correlations

For E-cadherin, positive immunostaining was observed on the membrane of cancer cells and the intercellular junctions. It was strongly expressed in the normal esophageal squamous tissues (95.2%), moderately expressed in the adjacent atypical hyperplastic epithelium (71.0%), and weakly expressed in the ESCC

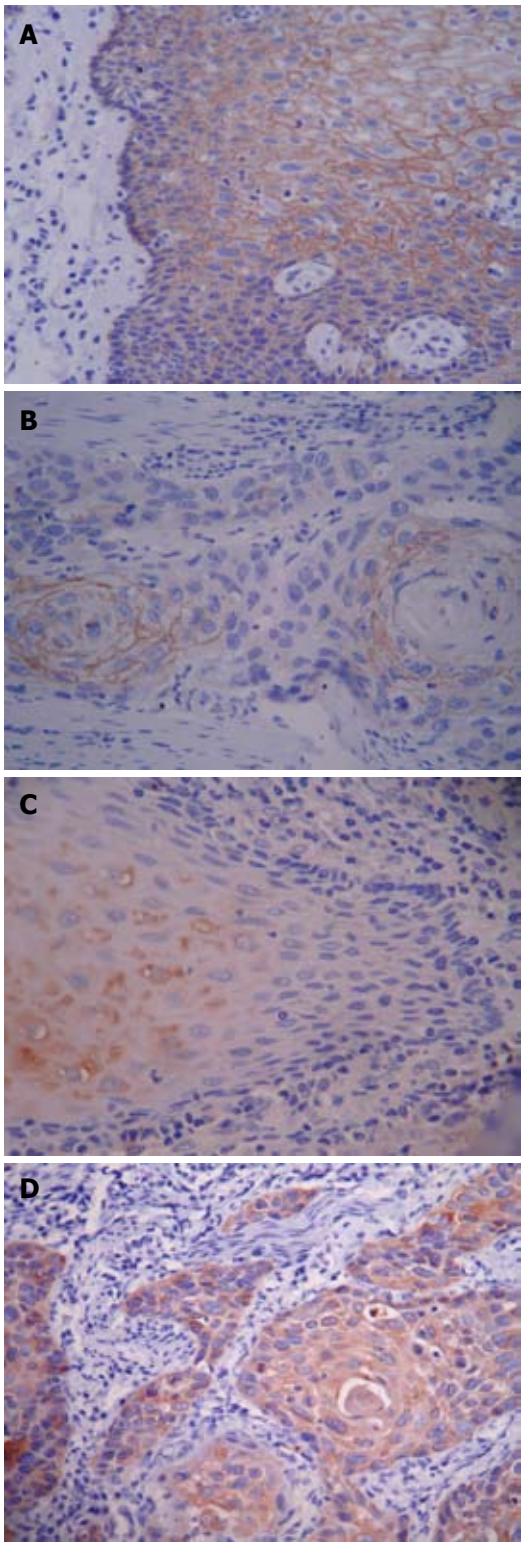

Figure 1 Expression of E-cadherin and N-cadherin in ESCC tissues and normal tissues. A: Strong expression (yellowish brown) of E-cadherin on the membrane of normal esophageal epithelial cells (PV, × 200); B: Weak expression (yellow) of E-cadherin on the membrane of ESCC cells (PV, × 200); C: Weak expression (yellow) of N-cadherin in the cytoplasm of normal esophageal epithelial cells (PV, × 200); D: Strong expression (yellowish brown) of N-cadherin in the cytoplasm of ESCC cells (PV, × 200).

tissues (40.3%) (Figure 1A, B and Table 2). Contrary to the E-cadherin, N-cadherin, which existed in the cytoplasm, was strongly expressed in the ESCC (75.8%)

Table 2 Correlations of E-cadherin, N-cadherin expression with clinicopathological features of ESCC

Items	*n*	E-cadherin			N-cadherin		
		Cases (%)	χ^2	*P*	Cases (%)	χ^2	*P*
Histological classification							
NEE	62	59 (95.2)			18 (29.0)		
AH	31	22 (71.0)	48.426	0.000	19 (61.3)	29.091	0.000
ESCC	62	25 (40.3)			47 (75.8)		
Histological grade							
I	15	11 (73.3)			8 (53.3)		
II	25	9 (36.0)	9.962	0.007	19 (76.0)	6.924	0.031
III	22	5 (22.7)			20 (90.9)		
Depth of invasion							
Not to serosa	7	6 (85.7)	4.797	0.029	2 (28.6)	6.916	0.009
To serosa	55	19 (34.5)			45 (81.8)		
Lymph node metastasis							
Yes	42	21 (50.0)	3.897	0.048	28 (66.7)	4.486	0.034
No	20	4 (20.0)			19 (95.0)		

NEE: Normal esophageal squamous tissues; AH: Adjacent atypical hyperplastic epithelium; ESCC: Esophageal squamous cell carcinoma tissues.

Table 3 Correlation between E-cadherin and N-cadherin protein expressions

	E-cadherin	N-cadherin (+)	N-cadherin (-)	γ	*P*
+	25	12	13	-0.534	0.000
–	37	35	2		

and moderately expressed in the adjacent atypical hyperplastic epithelium (61.3%), but weakly expressed in the normal esophageal squamous tissues (29.0%) (Figure 1C, D and Table 2). The correlations between the clinicopathological features and the expressions of E-cadherin and N-cadherin in the primary tumors are summarized in Table 2. Higher level of N-cadherin expression was significantly associated with higher histological grade, deeper invasion and more lymph node metastasis, while E-cadherin expression was associated with totally opposite sides.

Correlation between E-cadherin and N-cadherin protein expression

In order to know whether the expression of E-cadherin and N-cadherin in these tumors was associated, a crosstable analysis was performed (Table 3), which showed that their expression was significantly negatively correlated.

PT67 cells producing retrovirus

After G418 selection for 30 d, the stable colonies of pEGFP-MSCVneo plasmid (control vector) with viral titer 1×10^7 cfu/L and colonies of pMSCVneo/N-cadherin (RNAi vector) with viral titer 3×10^7 cfu/L were picked up to infect EC9706 cells (Figure 2A).

Establishment of transfected EC9706 cells

EC9706 cells were infected with the concentrated viral supernatant and selected with G418 as described above. After screening with G418 for 2 wk, the isolated G418-

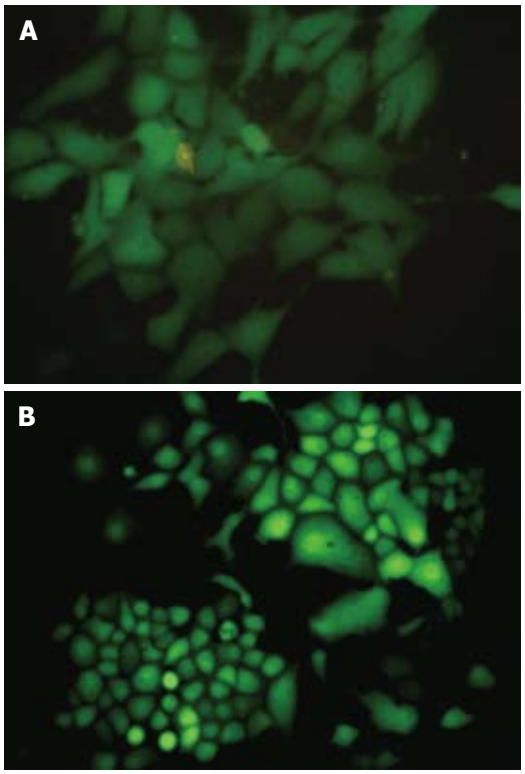

Figure 2 EGFP expression in PT67 cells and EC9706 cells after pMSCV-neo/N-cadherin transfection. A: Selected by 1000 mg/L G418 for 15 d and 300 mg/L G418 for 15 d, the PT-67 cells transfected with pMSCVneo/N-cadherin plasmid expressed EGFP stably. The figure was taken under fluorescence microscope (× 400, at 488 nm); B: Selected by 600 mg/L G418 for 14 d and 300 mg/L G418 for 10 d, the EC9706 cells infected with pMSCVneo/N-cadherin viral supernatant expressed EGFP stably. The figure was taken under fluorescence microscope (× 400, at 488 nm).

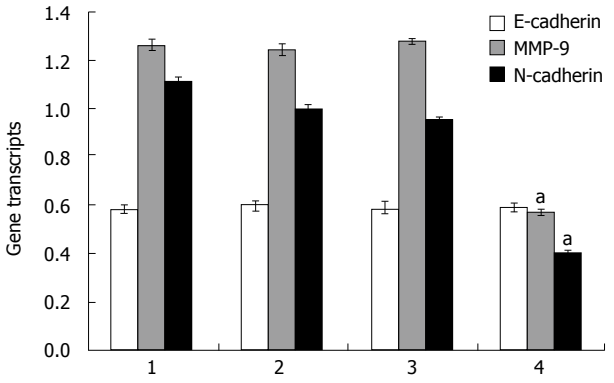

Figure 3 E-cadherin, N-cadherin and MMP-9 gene transcripts detected by real-time PCR. 1: Positive control; 2: Untreated EC9706 cells; 3: EC9706 cells with control vector; 4: EC9706 cells with N-cadherin RNAi. $^{a}P < 0.05$ *vs* 2 and 3.

resistant clones (Figure 2B) were transferred into larger culture vessels to expand for further experiments.

N-cadherin depletion affected expression of E-cadherin and MMP-9 by EC9706

To investigate the expression phenotypes of E-cadherin and MMP-9 when N-cadherin was down-regulated, real-time PCR and Western blotting were employed to examine the expressions of the N-cadherin, E-cadherin

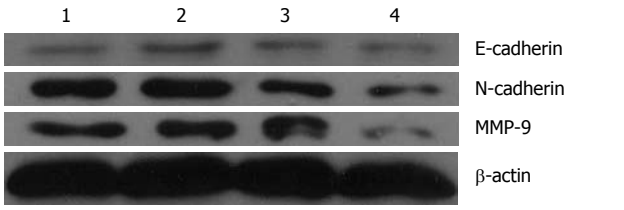

Figure 4 E-cadherin, N-cadherin and MMP-9 proteins detected by Western bloting. 1: Positive control; 2: Untreated EC9706 cells; 3: EC9706 cells with control vector; 4: EC9706 cells with N-cadherin RNAi. E-cadherin proteins were 0.247 ± 0.010, 0.252 ± 0.087, 0.249 ± 0.07 and 0.250 ± 0.006, respectively, from lane 1 to lane 4. $P > 0.05$. The N-cadherin proteins were 0.681 ± 0.003, 0.679 ± 0.004, 0.653 ± 0.009 and 0.342 ± 0.006, respectively, from lane 1 to lane 4. $P < 0.05$. The MMP-9 proteins were 0.624 ± 0.011, 0.628 ± 0.010, 0.623 ± 0.009 and 0.282 ± 0.010, respectively, from lane 1 to lane 4. $P < 0.05$.

and MMP-9 in ESCC cell line EC9706. Transfection of N-cadherin RNAi lowered N-cadherin mRNA level (0.397 ± 0.013) to less than 40%, compared with the untreated cells (1.000 ± 0.016) (Figure 3), and the N-cadherin protein (0.342 ± 0.006) to 50%, compared with the untreated cells (0.679 ± 0.004) (Figure 4).

The levels of MMP-9 mRNA and protein lowered by about 50% in the N-cadherin depleted cells compared with the untreated cells (Figures 3 and 4). The MMP-9 mRNA reduced from 1.241 ± 0.023 in the untreated cells to 0.566 ± 0.016 in N-cadherin RNAi cells, and the protein reduced from 0.628 ± 0.010 to 0.282 ± 0.010. However, it seems that N-cadherin shRNA did not affect the levels of E-cadherin mRNA and protein (Figures 3 and 4).

Knocking down N-cadherin decreased invasiveness of EC9706 in vitro

N-cadherin level was elevated with the malignancy in the ESCC tissues in a previous experiment. When N-cadherin was down-regulated, the migration of the cells was also reduced, compared with the untreated cells. Compared with the untreated EC9706 cells (Figure 5A) and the cells with control vector (Figure 5B), the N-cadherin negative-cells (Figure 5C) migration across the membranes decreased dramatically.

DISCUSSION

Cadherins are a class of type-1 transmembrane proteins. They are calcium dependent cell-cell adhesion glycoproteins. They play important roles in tissue formation and maintenance during embryonic development, and in the induction and maintenance of normal architecture and function in adult tissues. The most researched proteins of the cadherin family are E-cadherin and N-cadherin[8].

In the present research with ESCC, we found down-regulation of E-cadherin and increased N-cadherin. Normal E-cadherin expression contributes to the maintenance of epithelial integrity and polarized function[9,10]. Mutations in E-cadherin gene are correlated with gastric, breast, colorectal, thyroid and ovarian

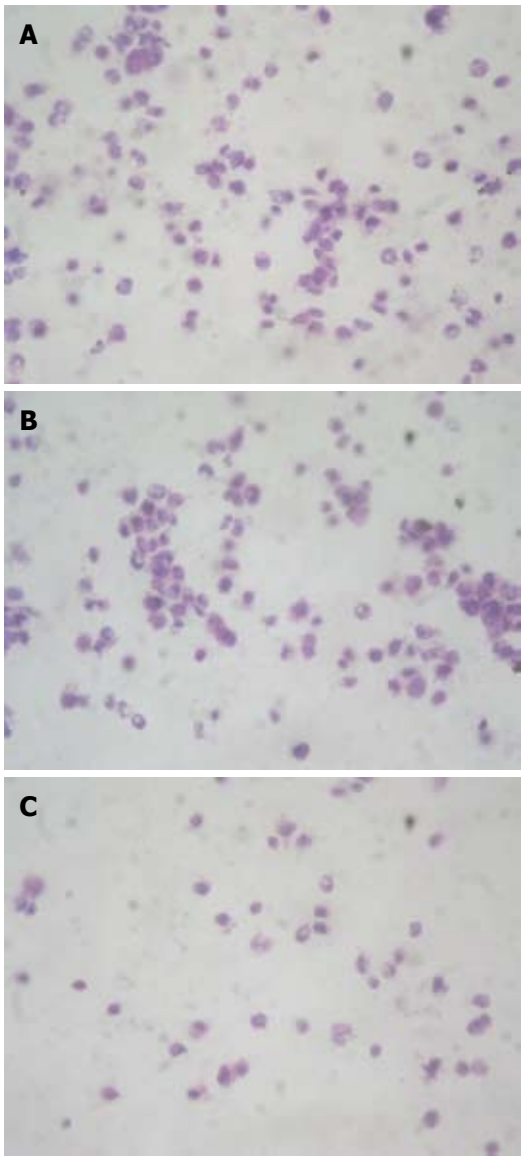

Figure 5 Migration assay in transwell chamber. A: Untreated EC9706 cells; B: EC9706 cells with control vector; C: EC9706 cells with N-cadherin RNAi. They were 123.40 ± 8.234, 126.00 ± 10.295 and 49.60 ± 6.804, respectively, from A to C. $P < 0.05$. (HE, × 200).

cancer[11,12]. Much lower levels were found to be present in the poorly differentiated lung cancer, indicating the worse prognosis[13]. Unlike E-cadherin, which is inversely correlated with invasiveness, N-cadherin may promote motility and invasion in carcinoma cells. N-cadherin has been shown to enhance cell migration during epithelial-mesenchymal transformation[14]. Aberrant N-cadherin expression was also found in breast carcinoma cells and prostate carcinoma cells[3,4]. In epithelial carcinoma, E-cadherin is down-regulated in most cases, sometimes accompanied by the up-regulation of another cadherin, for example, N-cadherin[15,16]. For the present research with ESCC, less E-cadherin and more N-cadherin were expressed in the ESCC tissue with deep invasion, poor differentiation and lymph node metastasis than with superfacial invasion, well differentiated and negative metastasis tissues. The N-cadherin expression was increased in the advanced ESCC tissues where E-cadherin was down-regulated, suggesting that they undergo a switch from E- to N-cadherin expression. The shift in expression from E- to N-cadherin and their mutually exclusive expression pattern in invasive tumor cell lines strongly reflect that the dedifferentiation from an epithelial to a mesenchymal phenotype was often associated with an increased invasive state[17]. The exact underlying mechanism has not been clear, but in many carcinomas, this "cadherin switch" was observed, especially in those where mild and non-progressive cells transformed into a more invasive phenotype[3,18,19]. Ras, Src, Rho, PI3K and Wnt signaling pathways were supposed to be involved in this switch[20-22].

We have proved that reduced E-cadherin and increased N-cadherin were present in advanced ESCC, and the following RNAi-mediated N-cadherin silence in EC9706 cell line further disclosed the correlation of E-cadherin and N-cadherin with ESCC progression. The down-regulation of N-cadherin did not change the expression of E-cadherin mRNA and its product. While N-cadherin and MMP-9 were reduced significantly in transcription level and translation level, less cells demonstrated invasiveness.

Local tumor invasion is characterized by at least two changes of function by the cancer cells. Firstly, these cells express higher levels of membrane-type and secreted proteolytic enzymes (e.g. the MMPs) in comparison with their normal epithelioid counterparts. Their contribution to invasion ranges from breakdown of the extracellular matrix, over-release of pro-invasive factors, to cleavage of cell-cell adhesion molecules[23]. Secondly, cancer cells are more motile than normal epithelial cells. Local tumor invasion is also made possible by disruption of epithelial cell junctions. E-cadherin, a part of the adherens junctions, plays an important role in maintaining the epithelioid cell organization and in preventing invasion[24]. Loss of function is thought to contribute to progression in cancer by increasing proliferation, invasion, and/or metastasis[5]. Forced expression of N-cadherin in well-differentiated breast cell lines did not change their E-cadherin expression as indicated, but stimulated marked increases in cell migration and invasion[25]. The ability of N-cadherin-expressing EC9706 cells to adhere to N-cadherin-expressing endothelial sheets may facilitate their transit through the vasculature and improve their ability to form metastasis. The present results also confirmed that the N-cadherin expression down-regulation did not affect E-cadherin mRNA and protein levels, but the invasiveness of all the EC9706 cells was weakened as compared with the untreated EC9706 cells. It might be postulated that N-cadherin, rather than E-cadherin, plays an important role in the cancer progression and metastasis.

Less invasiveness was shown in the N-cadherin-negative tumor cells. Therefore, knocking down N-cadherin can weaken the aggressiveness of cancer cells, by the mechanism involving more than a change in cellular adhesion. MMPs were thought to predominantly degrade structural components of the extracellular matrix

(ECM), thereby facilitating cell migration. In addition to cleaving structural ECM components, collagen type Ⅳ and the cell-adhesive molecules are also MMP substrates, increasing the invasive behavior of cells. Type I collagen is the predominant constituent of the perivascular ECM, and as mentioned previously, a variety of MMPs are capable of degrading collagen, including interstitial collagenase and neutrophil collagenase. The metastatic cells thus use these proteases to invade basement membrane and its underlying connective tissues and then subsequently through the basement membrane of the small blood vessels and lymphatics[26-30]. With RT-PCR and Western blotting, the level of MMP-9 mRNA and protein in the RNAi-mediated N-cadherin silencing EC9706 cells was found to be reduced as compared with untreated cells. It was supposed that the lower invasiveness of EC9706 cells was the consequence of down-regulation of N-cadherin, by the mechanism of decreasing the MMP-9 expression. The MMP-9 reduction resulted in less degradation of ECM, and thereby, the cancer cells were less aggressive. But which signaling pathway was involved in the N-cadherin to MMP-9 should be studied in the future researches.

In this study, decreased E-cadherin expression and increased N-cadherin expression were found more frequently in advanced ESCC than in low grade ESCC, confirming that the down-regulation of E-cadherin expression and up-regulation of N-cadherin expression were closely associated with the infiltration, invasion and metastasis of ESCC. *In vitro* experiments also demonstrated that even the E-cadherin mRNA and protein did not change much in the N-cadherin knocking down EC9706 cells, but the invasiveness of cancer cells was dramatically reduced. The decreased MMP-9 mRNA and its product were observed in the N-cadherin-negative cells, the majority of which lost their ability of migration *in vitro*. It was supposed that the N-cadherin played a role of facilitating cell invasion by MMP-9. In summary, our data suggest that N-cadherin is an important factor in the invasiveness of esophageal squamous cell carcinoma and N-cadherin may serve as a potential molecular target for biotherapy of esophageal squamous cell carcinoma.

COMMENTS

Background

Among the members of cadherin family, E-cadherin and N-cadherin have been extensively studied for their biological activities and associations with cancer cell invasion. Latest research on prostate cancer and breast cancer has proved that the up-regulated N-cadherin plays even more important roles in cell progression and metastasis.

Research frontiers

The shift in expression from E- to N-cadherin and their mutually exclusive expression pattern in invasive tumors strongly reflects dedifferentiation from an epithelial to a mesenchymal phenotype, often associated with an increased invasive state. This "cadherin switch" has been observed, especially in those where mild and non-progressive cells transformed into more invasive phenotypes. Therefore, many studies have focused on the exact underlying mechanism involved in this cadherin switch.

Innovations and breakthroughs

In the current study, the expression of N-cadherin and E-cadherin was first examined in esophageal squamous cell carcinoma (ESCC) specimens and the results revealed that increased expression of N-cadherin and decreased expression of E-cadherin were related to invasion, differentiation, and lymph node metastasis, the roles of N-cadherin in the invasiveness of ESCC were first investigated in the EC9706 cell line transfected by retroviral-mediated N-cadherin RNAi and the results revealed that N-cadherin knock-down significantly decreased the invasiveness of EC9706 cells.

Application

This study has indicated that N-cadherin is an important factor in the invasiveness of ESCC and N-cadherin may serve as a potential molecular target for biotherapy of ESCC.

Terminology

Cadherins are a class of type-1 transmembrane proteins. They are calcium-dependent cell-cell adhesion glycoproteins. They play important roles in tissue formation and maintenance during embryonic development, and in the induction and maintenance of normal architecture and function in adult tissues.

Peer review

The authors examined the expression pattern of N-cadherin and E-cadherin. They demonstrated that N-cadherin is an important factor in the invasiveness of ESCC and it may serve as a potential molecular target for biotherapy of ESCC.

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Epigenetics of proteasome inhibition in the liver of rats fed ethanol chronically

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Author contributions: Oliva J, Dedes J, and Li J performed the real-time PCR experiments, the microarray analysis, and the animal care and treatments, respectively; French SW provided the resources and facilities; Bardag-Gorce F designed the study, performed the nuclei and histone isolation, proteasome activity measurement, Western blot analysis, and wrote the manuscript.
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Abstract

AIM: To examine the effects of ethanol-induced proteasome inhibition, and the effects of proteasome inhibition in the regulation of epigenetic mechanisms.

METHODS: Rats were fed ethanol for 1 mo using the Tsukamoto-French model and were compared to rats given the proteasome inhibitor PS-341 (Bortezomib, Velcade™) by intraperitoneal injection. Microarray analysis and real time PCR were performed and proteasome activity assays and Western blot analysis were performed using isolated nuclei.

RESULTS: Chronic ethanol feeding caused a significant inhibition of the ubiquitin proteasome pathway in the nucleus, which led to changes in the turnover of transcriptional factors, histone-modifying enzymes, and, therefore, affected epigenetic mechanisms. Chronic ethanol feeding was related to an increase in histone acetylation, and it is hypothesized that the proteasome proteolytic activity regulated histone modifications by controlling the stability of histone modifying enzymes, and, therefore, regulated the chromatin structure, allowing easy access to chromatin by RNA polymerase, and, thus, proper gene expression. Proteasome inhibition by PS-341 increased histone acetylation similar to chronic ethanol feeding. In addition, proteasome inhibition caused dramatic changes in hepatic remethylation reactions as there was a significant decrease in the enzymes responsible for the regeneration of S-adenosylmethionine, and, in particular, a significant decrease in the betaine-homocysteine methyltransferase enzyme. This suggested that hypomethylation was associated with proteasome inhibition, as indicated by the decrease in histone methylation.

CONCLUSION: The role of proteasome inhibition in regulating epigenetic mechanisms, and its link to liver injury in alcoholic liver disease, is thus a promising approach to study liver injury due to chronic ethanol consumption.

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Key words: Alcohol liver injury; Betaine; Epigenetic mechanisms; Homocysteine methyltransferase; Proteasome inhibition; S-adenosylmethionine

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INTRODUCTION

A growing body of evidence indicates that specific histone modifications and modifying enzymes play essential roles in both global and tissue-specific chromatin organization[1]. Histone modifications, such as acetylation and methylation, play important roles in the regulation of gene expression, and impact many fundamental biological processes (i.e. in the cell cycle and cell proliferation). These modifications represent the inheritable epigenetic memory, which is transmitted with high fidelity to future cell generations.

Acetylation of histones is a major factor in

the regulation of chromatin remodeling and gene transcription. A sophisticated orchestra of proteins, such as histone acetyltransferases [HATs, gene activation (acetylation)], and histone deacetylases (HDACs), are required to regulate epigenetic mechanisms[2]. Acetylation of histone and non-histone protein is thus emerging as a central process in transcriptional activation, since nuclear HATs act as transcription co-activators, which have been shown to acetylate different transcriptions (i.e. p53, β-catenin, MyoD, SREBP-1)[3].

Alcohol consumption affects epigenetic mechanisms, and causes an increase in histone lysine acetylation, which has been associated with an increase in gene expression that may contribute to uncontrolled transcription[3]. These modifications are the results of changes in the activities of specific modifying enzymes that play essential roles in chromatin structure and specific gene expression. It has previously been shown that chronic ethanol feeding affects epigenetic mechanisms by causing an increase in the stability of histone-modifying enzymes, and thus, in histone modifications[4,5]. However, little is known about the mechanisms that control the life span of histone-modifying enzymes. For example, the consequence of proteasome inhibition in the nucleus, and its effects on epigenetic mechanisms, have not yet been investigated, and there is very little evidence on proteasome involvement in the turnover of the transcriptional factor and histone-modifying enzymes that regulate epigenetic mechanisms.

Our hypothesis is that proteasome inhibition, induced by ethanol feeding, is associated with histone modification, and is involved in the regulation of histone-modifying enzymes, such as the HAT p300.

The present study demonstrates the role of proteasome activity in epigenetic mechanisms, which significantly contribute to liver injury due to chronic ethanol feeding. Proteasome proteolytic activity regulates histone modifications by regulating the recruitment and stability of histone-modifying enzymes in the nucleus, and, therefore, regulates the chromatin structure, allowing easy access to chromatin by RNA polymerase and enhanced gene expression. The proteasome activity is also believed[6] to be critical for the expression of certain genes, such as those of the enzymes responsible for hepatic transmethylation reactions. In this study, microarray analysis showed up-regulation and down-regulation of a large number of genes, indicating that proteasome activity is essential for up-regulation, as well as down-regulation of specific gene expression. Proteasome inhibition caused a decrease in the gene expression of several enzymes involved in methionine metabolism, particularly betaine-homocysteine methyltransferase (BHMT), which was significantly down-regulated when the proteasome was inhibited[7]. These results indicated that DNA and histone methylation, which play important roles in the regulation of gene silencing, may be affected by proteasome inhibition, and, therefore, may impact many fundamental biological processes.

Previous reports have shown that gene expression changes were numerous at high levels of ethanol, when compared to their pair-fed controls. 1300 genes were changed[8-10]. Similarly, a preliminary microarray analysis of the livers of rats given the proteasome inhibitor PS-341, has shown marked changes in gene expression. Thus, a question arose: which mechanism is involved in this large number of gene expression changes? We believe that this mechanism is epigenetic in nature.

We believe that these modifications in gene expression are the result of a decrease in the activity of the proteasome in the nucleus, which would, for instance, increase the p300 HAT level and activity. Since p300 is responsible for a broad range of gene regulation, the activation of p300 acetylation in histones will increase and activate the expression of a large number of genes in a nonspecific and reversible manner.

The role of the proteasome in regulating histone methylation is also critical because the expression of several genes was changed when the proteasome was inhibited. We have previously demonstrated that proteasome inhibition causes a downregulation in the expression of several genes[8], particularly the gene involved in the remethylation pathway. The study of this remethylation, particularly, the reactional mechanism that regenerates S-adenosylmethionine (SAMe), the major methyl donor, is essential because this is the system that transfers the methyl group to DNA, histones and non-histone proteins *via* the methyltransferases, such as glycine N-methyltransferase (GNMT).

In this study, BHMT gene expression was markedly decreased by proteasome inhibition. BHMT is an essential enzyme in the remethylation pathway, and is involved in the recovery of SAMe. Betaine, a choline derivative which has been used clinically to treat, with some success, patients with methylenetetrahydrofolate reductase deficiency[11,12], acts as a substrate for BHMT, and serves as an alternative methyl donor for remethylation of homocysteine in the liver and kidney[13]. Therefore, betaine supplementation may cover the down-regulation of gene expression induced by proteasome inhibition, and correct the deregulation of hepatic transmethylation reactions due to the proteasome inhibition-induced decrease in BHMT activity.

MATERIALS AND METHODS

Animals

Male Wistar rats (Harleco, Hollister, CA, USA), weighing 250-300 g, were fed ethanol using the Tsukamoto-French intragastric model[14,15]. PS-341 was administered intraperitoneally, 24 h before sacrifice[16,17]. The rats were maintained according to the Guidelines of Animal Care, as described by the National Academy of Sciences and published by the National Institute of Health (1996).

Nuclei isolation

Histones were isolated from the nuclei, according to the method of Umlauf *et al*[18]. Liver tissues, frozen in isopentane and immersed in liquid nitrogen, were homogenized in a Dounce homogenizer with 10 strokes.

Homogenates were centrifuged for 10 min at 6000 × *g*. Pellets were resuspended, placed on ice for 10 min, and then centrifuged for 20 min at 9000 × *g* on a sucrose cushion. The pellets contained the nuclei. Histones were isolated from the nuclei, according to the method of Shechter *et al*[19]. Isolated nuclei were mixed with 0.2 mol/L H_2SO_4, and incubated on a rotator for 30 min at 4℃. Samples were spun in a microcentrifuge at 16 000 × *g*, for 10 min. Dissolved histones in the supernatant were precipitated with 33% TCA. After acetone wash, histones were dissolved in an appropriate buffer, and further analyses were carried out.

Proteasome chymotrypsin-like activity assay

Nuclei were isolated as mentioned above, and 1 μg of total protein was used. The reaction mixture contained 50 mmol/L Tris–HCl pH 8, 1 mmol/L DTT, and 40 μmol/L Suc-LLVY-AMC substrate for chymotrypsin-like activity. The mixture was incubated for 30 min at 37℃, and the reaction was then stopped by adding 100 μmol/L monochloroacetate and 30 mmol/L sodium acetate (pH 4.3). Fluorescence was determined by measuring the release of AMC (λ excitation: 370 nm, λ emission: 430 nm) using a Perkin Elmer LS 30 spectrofluorometer.

Western blot analysis

Proteins (50 μg) from isolated nuclei or isolated histones were separated by SDS-PAGE, and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) for 1 h in 25 mmol/L Tris-HCl (pH 8.3), 192 mmol/L glycine and 20% methanol. The membranes were stained using primary antibodies to the antigens. The appropriate species anti-polyclonal and anti-monoclonal HRP-conjugated antibodies were used as secondary antibodies. The membranes were subjected to chemiluminescence detection using luminal, according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Microarray analysis

Fast frozen rat liver tissue was subjected to microarray analysis. Total liver RNAs were extracted with UltraspecTM RNA Isolation Systemic (Biotecx Laboratories, Houston, TX, USA), and cleaned with Rneasy columns (Qiagen, Valencia, CA, USA). Five micrograms of total RNA were used for preparing biotin-labeled cRNA. Labeled and fragmented cRNA was subsequently hybridized to Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA). Labeling, hybridization, image scanning, and initial data analysis were performed at the Microarray Core at Los Angeles Biomedical Research Institute. Sample preparation and loading, hybridization, staining, and microarray data analysis were then performed[8].

Quantitative RT-PCR

Total liver RNAs were extracted with Trizol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNAs was performed with 5 μg total RNA, and 50 ng random hexamer primers using SuperScriptIII RNase H-Reverse Transcriptase (Invitrogen). PCR primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA). The primers for rat p300 are shown in Table 1.

Table 1 List of primer sequences used in RT-PCR

p300	XM_576312	Forward	5GAGGTCACTGTTCGGGTTGTTC
p300	XM_576312	Reverse	5TGGTTCGATATGGAAAAGATTCTG
BHMT	NM_030850	Forward	5GGGCAGAAGGTCAATGAAGCT
BHMT	NM_030850	Reverse	5ACCAATGCATCCCCTTCGT

Quantitative PCR was achieved using the SYBR Green JumpStartTM (Applied Biosystems). Thermal cycling consisted of an initial step at 50℃ for 2 min, followed by a denaturation step at 95℃ for 10 min, and then 40 cycles at 95℃ for 15 s and 60℃ for 1 min. A single PCR product was confirmed with the heat dissociation protocol at the end of the PCR cycles. Each data point was repeated three times.

Sense and anti-sense: quantitative PCR was achieved using the SYBR Green JumpStart™ Tag ReadyMix (Sigma, St. Louis, MO, USA) on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems). The thermal cycling consisted of an initial step at 50℃ for 2 min, followed by a denaturation step at 95℃ for 10 min, then 40 cycles at 95℃ for 15 s and 60℃ for 1 min. A single PCR product was confirmed with the heat dissociation protocol at the end of the PCR cycles. Quantitative values were obtained from the threshold PCR cycle number (Ct) at which point the increase in signal associated with an exponential growth for the PCR product was detected. The target mRNA abundance in each sample was normalized to its 18S level as $\Delta Ct = Ct_{\text{target gene}} - Ct_{18S}$. For each target gene, the highest ΔCt was assigned as ΔCt_{max}.

Statistical analysis

Data were obtained from at least three animals in each group. Bars represent mean ± SE. *P* values were determined by one-way ANOVA and Student-Newman Keuls for multiple group comparisons (Sigma-Stat software, San Francisco, CA, USA). $P \leqslant 0.05$ was used to establish significant differences. Correlation of data was done by linear regression analysis using Pearson's period momentum method.

RESULTS

Microarray analysis of liver samples from rats fed ethanol showed that a large number of genes (about 1300) were up-regulated and down-regulated due to chronic ethanol feeding[8]. Microarray analyses of liver samples from rats given PS-341 (Bortezomib, Velcade®) also showed dramatic changes in gene expression (about 2082 genes changed) affecting several functional pathways (Figure 1).

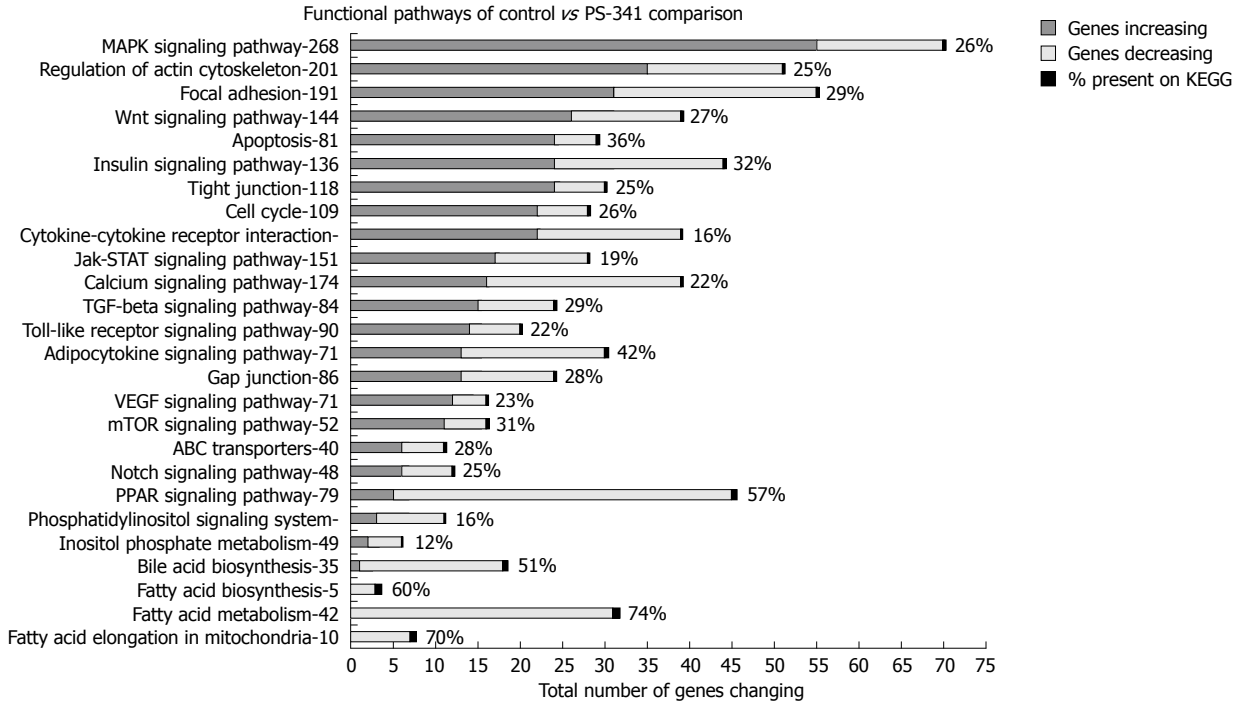

Figure 1 Kegg functional pathway changes in gene expression induced by proteasome inhibition. Both ethanol feeding and proteasome inhibition affected almost all pathways.

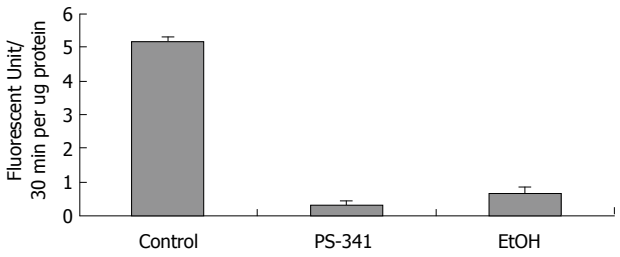

Figure 2 Nuclear proteasome chymotrypsin-like activity. 20S proteasome chymotrypsin-like activity was measured in isolated nuclei from the liver of rats fed ethanol chronically and from the liver of rats given PS-341.

The present study was based on the observation that the inhibition of proteasome, caused by chronic ethanol feeding, participated in the development of liver injury due to ethanol by altering the mechanisms through which normal epigenetic regulation occurs. The consequence of this was a marked change in the gene expression of several functional pathways in liver cells (Figure 1).

Data mining and gene specific pathway clustering showed that, similar to ethanol feeding, several transcriptional factors, such as cell cycle, histone modifying enzymes, and the remethylation pathway, were significantly changed by proteasome inhibition. Proteasome inhibition by PS-341 thus proved to be a powerful tool to investigate the role of proteasome activity in epigenetic mechanisms.

To verify the hypothesis that gene expression changes are regulated by nuclear proteasome activity, where inhibition is caused by chronic ethanol feeding[20], proteasome activity was measured in isolated nuclei from the liver of rats fed ethanol chronically, and from the liver of rats given PS-341. Figure 2 shows that chronic ethanol feeding caused a significant decrease in proteasome chymotrypsin-like activity in isolated liver nuclei.

To further investigate the role of proteasome activity in regulating epigenetic mechanisms, histone acetylation was analyzed in the liver of rats given PS-341, and compared to histone acetylation in the liver of rats fed ethanol chronically. Figure 3A shows that acetylated histone 3 lysine 9 (AcH3K9) was increased in the liver of rats fed ethanol, and that acetylated histone 3 lysine 27 (AcH3K27) was increased in the liver of rats given PS-341 (Figure 3B).

Increased acetylation was concomitant with an increase in the level of HAT p300, which was linked to significant proteasome inhibition shown in the liver of rats fed ethanol chronically (Figure 3D), and in the liver of rats given PS-341 (Figure 3C). P300 was increased in the ethanol-isolated nuclear extract, which confirmed a previous report[4]. It is now well established that ethanol feeding increases histone acetylation[4,5], which correlates with an increase in the acetyltransferase CBP/p300, and a decrease in Sirt1 activity[3,21]. Under our experimental conditions, Sirt1 gene expression and protein level were up-regulated[22], or showed no significant changes[4]. However, the activity of the enzyme may be decreased due to the low level of NAD+, a Sirt1 cofactor[23].

The increased level of histone acetylation substantiated the increase in p300 activity in the liver of rats fed ethanol. In addition, proteasome inhibition by PS-341 caused a significant increase in histone acetylation, which substantiated our hypothesis that

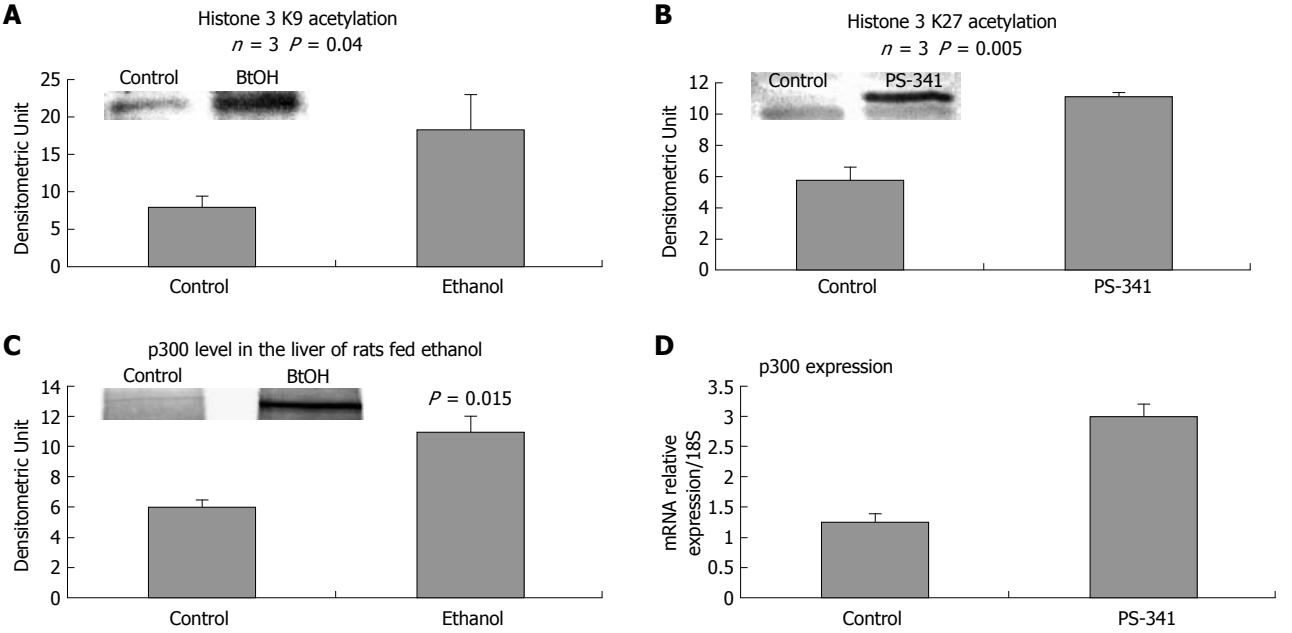

Figure 3 Role of proteasome inhibition in histone acetylation. A: Proteasome inhibition caused a significant increase in histone acetylation as shown in the liver nuclear extracts of rats fed ethanol and in the liver nuclear extracts of rats given PS-341(B). C: p300 protein level was increased in the nuclear extract from the livers of rats fed ethanol chronically, and in the liver nuclear extracts of rats given PS-341, as shown by real time PCR (D), $P = 0.003$. (mean ± SE, $n = 3$).

ethanol induced-histone acetylation is associated with proteasome inhibition. The level of p300 was increased in the nucleus when ethanol was fed chronically, and may also have accumulated due to proteasome proteolysis slowdown in the nucleus.

Proteasome activity has also been found to be involved in the regulation of remethylation enzyme gene expression, thus affecting DNA and histone methylation when it is inhibited. Microarray data mining showed a down-regulation in gene expression of the remethylation enzymes when the proteasome was inhibited (Figure 4A), especially BHMT, which reconstitutes methionine, the major element in the methylation pathway (Figure 4B). Mat1a, Adm (S-adenosylmethionine decarboxylase), and Ahcy (S-adenosylhomocysteine hydrolase) were also down-regulated by proteasome inhibition (Figure 4A), indicating the role of proteasome activity in the methionine-metabolizing enzymes system. Figure 4C shows that proteasome inhibition significantly decreased the protein level of BHMT in the liver of rats given PS-341, and, to a lower extent, in rats subjected to chronic ethanol feeding. These findings demonstrated the role of the proteasome in regulating gene expression in the remethylation pathway, and the role of proteasome activity in the cellular remethylation pathway. Western blot analysis showed a significant decrease in H3K9 dimethylation in the liver of rats given PS-341. A decrease in H3K9 dimethylation (Figure 4D) was also observed in ethanol-treated cells[24], suggesting similar effects of proteasome inhibition and ethanol feeding. These results showed for the first time the role of proteasome activity in regulating the mechanisms of cellular remethylation, and is a promising approach in chemotherapy regimens which use proteasome inhibitor as an anti tumor drug.

DISCUSSION

Several studies have shown that the inhibition of the ubiquitin-proteasome pathway is a pathobiological mechanism associated with the development of liver disease, especially alcoholic liver disease[25,26]. We believe that ethanol-induced inhibition of proteasome activity may play a significant role in the deregulation of epigenetic mechanisms and the mechanism related to liver injury in alcoholic liver disease (ALD).

Many cellular signaling pathways are controlled by selective proteolysis of key regulatory proteins via the ubiquitin-proteasome system[4,12,27]. Proteasome is involved in RNA polymerase Ⅱ degradation[28], which is a key step in controlling the transcriptional mechanism, preventing uncontrolled transcription that may occur when ethanol metabolism generates oxidative stress, which causes DNA damage[29]. Reports have shown that proteasome dysfunction leads to apoptotic death of hepatocytes and sensitization to tumor necrosis factor (TNF) cytotoxicity, leading to direct hepatic injury[25]. Although it is now evident that inhibition of cytoplasmic proteasome function is consistently shown in ALD models, the way in which proteasome dysfunction may enhance hepatotoxicity is not well defined. In addition, the effects of ethanol feeding on the activity of nuclear proteasome and the consequences of proteasome inhibition on changes in epigenetic mechanisms have not yet been demonstrated. Most importantly, our previous investigations have repeatedly shown that chronic ethanol feeding causes significant proteasome inhibition in the cell[30,31]. The ubiquitin-proteasome pathway is the cellular proteolytic pathway dedicated to controlling protein stability, and understanding the link between the ubiquitin-proteasome pathway and the histone-

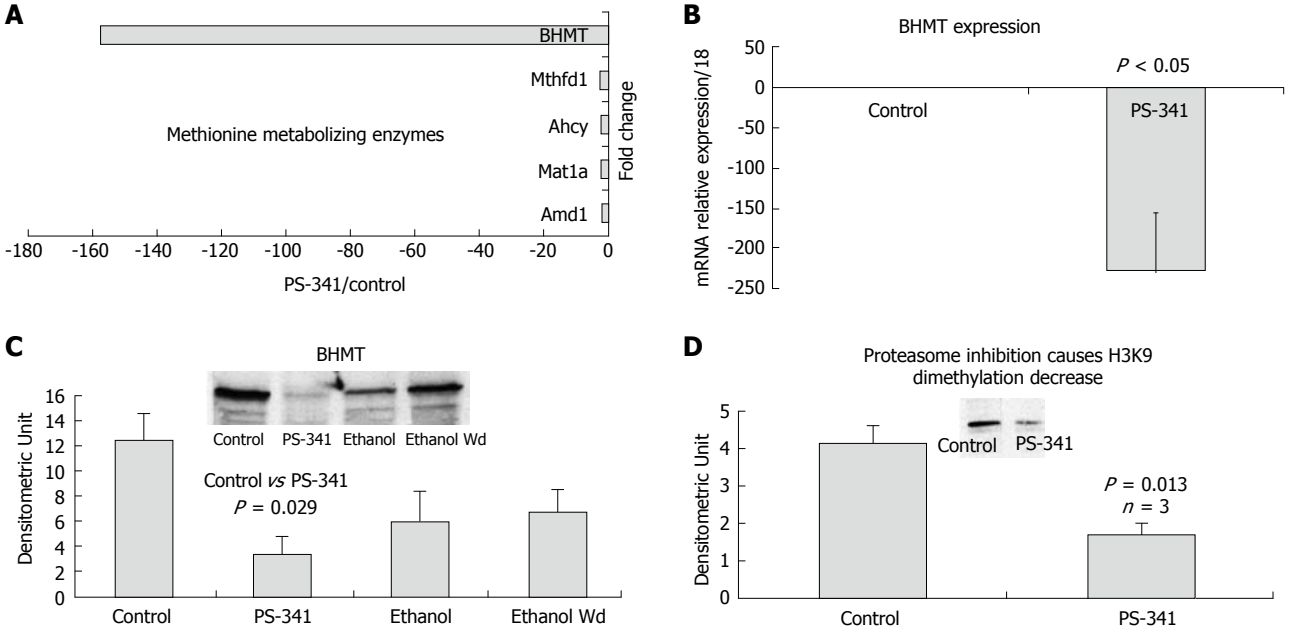

Figure 4 Role of proteasome inhibition in the remethylation pathway. A: Proteasome inhibition by PS-341 (PS) markedly decreased BHMT gene expression; B: Real time PCR analysis of BHMT expression; C: Western blot analysis of BHMT level in the liver of rats fed ethanol chronically and the liver of rats given PS-341. Note that the BHMT level was significantly reduced in the liver of rats treated with PS-341; D: Proteasome inhibition caused a decrease in histone methylation.

modifying machinery will define the link between proteasome proteolytic activity, epigenetic mechanisms, and the effect of toxic substances, such as ethanol and its metabolism generated end products, in the regulation and control of epigenetic mechanisms.

As predicted, our results showed an increase in acetylation of H3K9 in the liver nuclear extracts of rats fed ethanol chronically. This increase was associated with an increase in HAT p300, which confirmed a previous report[4]. In addition, p300 activation and histone acetylation were also obtained when proteasome activity was inhibited using PS-341, which supported the role of proteasome activity in regulating the stability of p300 in the nucleus and therefore supported the role of proteasome in regulating the acetylation mechanisms and thus gene expression. These results corroborate the findings of Marcu *et al*[32], which showed that p300 is a proteasome substrate, and that p300 is accumulated when the proteasome is inhibited.

The modification of histones mirrors the sophisticated protein machinery that controls gene expression and regulates transcription. Therefore, it would be naïve to suggest that, for instance, only H3K9 acetylation explained all the changes in the observed gene expression[11]. The balance between all histone lysine residue modifications accounted for the pattern of gene expression and further histone modifications are certainly involved in defining specific gene expression.

Histone methylation also plays a critical role in regulating gene expression and transcription. The cellular remethylation pathway is the major player in the methylation mechanism, because it produces the methyl donor SAMe. It is well known that chronic ethanol ingestion causes a serious deregulation of this pathway. Methionine adenosyltransferase 1-alpha, which is responsible for the conversion of methionine to SAMe, is decreased in the liver of rats fed ethanol[33]. Since SAMe is thus decreased, the level of DNA and protein methylation is decreased[34], and the level of homocysteine is increased[35]. The ratio SAMe/SAH (S-adenosylhomocysteine) is critical in the cell because it controls most of the methyltransferase activity. BHMT, which hydrolyzes betaine, helps remove SAH and homocysteine with subsequent regeneration of the methyl group and a reduction in the level of SAH. Our results show that BHMT was significantly down-regulated when the proteasome activity was inhibited, either by chronic ethanol feeding or by treatment with a proteasome inhibitor. In addition, there was a decrease in histone methylation, reflecting the role of proteasome activity in regulating the methylation mechanisms in the liver cell. Proteasome inhibition plays a critical role in regulating the gene expression of key enzymes in the remethylation pathway, such as BHMT.

COMMENTS

Background

Alcohol ingestion causes alterations in several cellular mechanisms, and leads to inflammation, apoptosis, and fibrosis. These phenomena are associated with significant changes in epigenetic mechanisms and with a subsequent liver cell memory. The ubiquitin-proteasome pathway is a vital cellular pathway which undergoes dysfunction due to chronic ethanol consumption.

Research frontiers

Although inhibition of proteasome function has been widely reported in models of alcoholic liver disease (ALD), why proteasome dysfunction may enhance hepatotoxicity is not well defined. In addition, there is no evidence of the effect of ethanol feeding on the activity of nuclear proteasome and the consequences of proteasome inhibition in epigenetic mechanisms and DNA repair.

Innovations and breakthroughs

The present study focused on the role of proteasome activity in gene expression and the effects of proteasome inhibition in changing epigenetic mechanisms.

The model used to study the consequences of proteasome inhibition due to chronic intragastric tube ethanol administration was proteasome inhibition by PS-341, a dipeptide boronic acid currently used in clinical trials as an anti tumor drug, and is associated with profound side effects. Inhibition of proteasome activity occurred in the nucleus of liver cells taken from rats fed ethanol chronically and from rats given PS-341. This inhibition caused changes in the turnover of transcriptional factors, histone modifying enzymes, and, therefore, affected epigenetic mechanisms. Histone acetylation was increased following both treatments and gene expression was changed. Identification of DNA and histone modifications was critical in regulating gene expression, especially genes involved in the cell cycle and apoptosis, and those involved in the metabolism of ethanol. In addition, proteasome inhibition has been shown to significantly affect the hepatic remethylation pathway. In particular, proteasome inhibition caused a decrease in gene expression of the enzyme betaine-homocysteine methyltransferase (BHMT), which is involved in the recovery of s-adenosylmethionine (SAMe). Histone methylation was also decreased when the proteasome was inhibited suggesting that hypomethylation was associated with the decrease in proteasome activity.

Applications

The phenomenon of hypomethylation is currently corrected by diet supplementation with a methyl donor (betaine) to redirect methionine/homocysteine metabolism toward recovery of methionine, and SAMe regeneration.

Terminology

Chronic ethanol feeding causes proteasome inhibition, which leads to cellular dysfunction including the accumulation of damaged proteins which form Mallory bodies in the liver of severe alcoholic patients, immune response deficiency, cell cycle deregulation and incorrect gene expression. The mechanism of incorrect gene expression was the focus of this study, particularly the consequences of ethanol induced-proteasome inhibition in the nucleus and the role played by proteasome in regulating epigenetic mechanisms.

Peer review

The impact of this study is great because when epigenetic mechanisms associated with proteasome inhibition are fully identified, a therapeutic approach will be initiated to counteract the ethanol induced-epigenetic changes, and prevent any cellular memory for future cell generations.

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Systemic chemotherapy for hepatocellular carcinoma in non-cirrhotic liver: A retrospective study

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Abstract

AIM: To investigate the efficacy and toxicity of systemic chemotherapy in a retrospective study of patients with hepatocellular carcinoma (HCC) occurring in normal or fibrotic liver without cirrhosis.

METHODS: Twenty-four patients with metastatic or locally advanced HCC in a normal or a fibrotic liver were given systemic chemotherapy (epirubicin, cisplatin and 5-fluorouracil or epirubicin, cisplatin and capecitabine regimens). Tumor response, time to progression, survival, and toxicity were evaluated.

RESULTS: There were 7 women and 17 men, mean age 54 ± 10 years; 18 patients had a normal liver and 6 had a fibrotic liver (F1/F2 on biopsy). Mean tumor size was 14 cm, 5 patients had portal vein thrombosis and 7 had metastasis. Patients received a median of 4 chemotherapy sessions. Overall tolerance was good. There were 5 partial responses (objective response rate = 22%), and tumor control rate was 52%. Second line surgical resection was possible in two patients. Median survival was 11 mo, and 1- and 2-year overall survival rates were 50% ± 10% and 32% ± 11%, respectively.

CONCLUSION: In patients with HCC in a non-cirrhotic liver, chemotherapy was well tolerated and associated with an objective response rate of 22%, including two patients who underwent secondary surgical resection.

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Key words: Antineoplastic protocols; Chemotherapy; Hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer, the fifth most common malignancy worldwide, and the third most common cause of cancer deaths[1]. Unfortunately, most patients are seen when the disease has reached a stage beyond curative treatment (surgery or percutaneous ablation), leaving palliative care as the only alternative. Based on the Barcelona-Clinic Liver Cancer (BCLC) staging classification[1] and treatment schedule, chemoembolization is the best option for intermediate stage patients, while for advanced stage patients, no standard treatment was established until 2007. Fortunately, after the positive results of the SHARP trial[2], a new treatment, sorafenib, was approved for advanced stage patients because of major improvements in overall survival and time to progression in comparison with placebo. In contrast to studies of most other malignancies, the efficacy of systemic chemotherapy has never been demonstrated in HCC. Despite the lack of demonstrated efficacy, doxorubicin is accepted by some physicians as a possible treatment for advanced HCC. In some recent trials using doxorubicin as a control arm, the investigational arms, a combination of platinum, doxorubicin, 5-fluorouracil and interferon in one trial[3] or nolatrexed[4], a novel thymidylate synthase inhibitor

in another trial, did not demonstrate any advantage or were associated with worse overall survival than doxorubicin alone. In one trial, this could have been partly due to toxicity, particularly because of hepatitis B virus reactivation[5]. Most likely, systemic chemotherapy lacks efficacy because of the frequently observed multidrug tumor resistance[6-8] (P-glycoprotein overexpression, p53 gene mutations), although greater drug toxicity related to the underlying cirrhosis might certainly have had an effect. In a search for clues to resolve this question, we retrospectively analyzed our records of cirrhosis-free HCC patients who received a standard chemotherapy regimen {ECF [epirubicin, cisplatin (CDDP) and 5-fluorouracil (5FU)][9] or ECC (epirubicin, CDDP and capecitabine)[10]}. Our goal was to determine whether chemotherapy was more effective in these patients than in HCC patients with cirrhosis.

MATERIALS AND METHODS

Between July 1999 and June 2006, we delivered standard chemotherapy regimens to 30 patients with HCC occurring in a non-cirrhotic liver. There was no indication for curative surgery or palliative treatment in these patients who had good performance status (ECOG PS 0 or 1), preserved liver function, normal blood cell counts (neutrophils > 15 000/mm^3, platelets > 100 × 10^9/L), normal renal function (serum creatinine < 110 μmol/L), and a measurable tumor target. Biopsy specimens, from the tumor and unaffected liver tissue, provided the diagnosis of HCC in non-cirrhotic liver in all 30 patients. None had a fibrolamellar cancer. The diagnosis of a normal liver was, however, less than certain in one patient since tumor cells had invaded most of the "normal liver" biopsy specimen. Five of the 30 patients had received a chemotherapy regimen other than ECF or ECC: capecitabine plus oxaliplatin (n = 3), and gemcitabine plus oxaliplatin (n = 2). Excluding these 6 patients, we thus retrospectively analyzed the records of 24 patients who received an ECF or ECC regimen for HCC occurring in a non-cirrhotic liver (normal liver or F1-F2 fibrosis); the ECF regimen was given from 1999 to 2002 and then we used the ECC regimen, which was much more convenient because it could be given orally.

The ECF treatment schedule was: epirubicin 60 mg/m^2 on day 1, CDDP 50 mg/m^2 on day 1, and 5FU 200 mg/m^2 administered in a continuous infusion from day 1 to day 21; in the ECC regimen, 5FU was replaced by capecitabine 1000 mg/m^2 twice a day from day 1 to day 14 followed by a 7-d off period. Courses were repeated every 21 d.

Tumor response was assessed with computed tomography performed before treatment onset and then every 9 wk (three chemotherapy courses). The tumor response (World Health Organization criteria) was considered as complete in the case of total disappearance of all tumors with normalization of α-fetoprotein (AFP) level, as partial for a decrease of tumor size less than 50%, and as stable disease with absence of progression and a decrease in tumor size less than 50%. Progression was an increase in tumor size greater than 25%. An objective response was defined by achievement of a complete or partial response. Patient tolerance was assessed using NCI-CTC AE version 2.0. In some patients with an excellent general status, second line therapy was proposed in the event of disease progression. Patient survival could be precisely evaluated for all patients and was calculated using the Kaplan-Meier method. The cause of death was recorded when available.

RESULTS

The ECF protocol was given to 10 patients, and the ECC protocol to 14. There was no difference in disease status between these two groups. The population was composed of 17 men and 7 women, mean age 54.3 ± 10.7 years (range: 23-77 years). The liver tumors were revealed in all patients by tumor-related complications (pain, fever, constitutional syndrome). The tumor developed in a non-cirrhotic liver in 18 patients and in a fibrotic liver in 6 (classified as F1 or F2). The etiological investigation revealed that 6 patients drank more than 4 units of alcohol per day, one had serological markers of hepatitis C, 2 had steatosis and were overweight, and one had genetic hemochromatosis treated by phlebotomy and was without iron liver overload on liver biopsy; no contributory factor was recognized in 14 patients. Among the 6 patients with minor fibrosis, 3 were drinkers, one had hepatitis C virus infection, one had genetic hemochromatosis and one had steatosis.

There was a unique tumor in 14 patients, 2-4 lesions in 6 and multiple or diffuse tumors in 4. Mean tumor size was huge: 14 ± 7 cm (range: 6-29 cm). Portal vein thrombosis was found in 5 patients and metastases in 7 (bones, lungs, lymph nodes). In the BCLC staging, all these patients could be classified as having an "advanced HCC", and in cases of unique tumor, tumor size was > 75% of the liver volume and surgery or transarterial chemoembolization could not be considered. Following Child-Pugh classification, and despite the absence of cirrhosis, all these patients were considered as Child class A and, using the CLIP system, 8 patients had 4 points, 10 had 3 points, 5 had 2 points and only one had 1 point (but this patient was metastatic).

None of these patients had previously received treatment for HCC. The patients received from one to 12 courses of the ECF/ECC regimen (median = 4). As the best response, 5 patients had a partial response (objective response rate 22%; 95% CI: 6-38%), 8 patients had stable disease (30%) and 11 had tumor progression; the disease control rate was then 52%. The median time to progression was 6 mo. At the cut-off date of March 15, 2007, 18 patients had died due to progression of their tumor and 6 patients were still alive; median survival time was 11 mo (range 3-90 mo). Actuarial overall survival rates (mean ± SD) at 6 mo, 1 year and 2 years were 71% ± 9%, 50% ± 10% and 32% ± 11%, respectively (Figure 1). The 5 patients with an objective response achieved prolonged survival: 3 died 13, 27 and 48 mo after treatment onset and 2 were still alive at the cut-off date. Surgical resection was undertaken in one

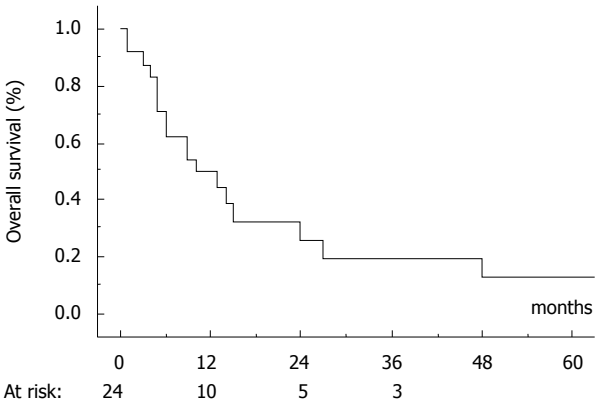

Figure 1 Overall survival in the population of 24 HCC patients with non-cirrhotic liver treated by an ECC/ECF regimen.

Table 1 Adverse effects in this series of 24 patients with HCC in non-cirrhotic liver treated by chemotherapy (ECC regimen)

Adverse effets	Grade 1-2	Grade 3-4 (%)
Alopecia	8	-
Neutropenia	4	4 (17)
Mucositis	4	0
Diarrhea	2	0
Renal failure	1	0
Asthenia	2	0
Hand-foot syndrome	5	0
Nausea, vomiting	12	2 (4)
Anemia	2	0
Coronary spasm	0	1

Percentages not given in this column

One patient died suddenly during the first course of chemotherapy.

patient 10 mo after treatment onset as a result of an objective response. This patient was alive at 90 mo with progression (the same chemotherapy was successfully resumed; she was subsequently treated with sorafenib). The other prolonged survivor had a 22-cm tumor; she underwent surgical resection when a minor response was observed after 9 courses of ECC. She was still alive and disease-free 36 mo after surgery. Initially, these 2 patients had a unique tumor involving more than 75% of the liver without portal vein thrombosis or extrahepatic metastases; both had an AFP level above 500 ng/mL; they were considered as CLIP 3.

Toxicity was graded using NCI-CTC criteria version 2.0. The main toxic effects are summarized in Table 1. Overall tolerance was good. Signs of grade 3-4 toxicity developed in 7 patients (29%), neutropenia in 4 (no cases of febrile neutropenia), gastrointestinal disorders (nausea, vomiting) in 2; one patient died suddenly after the first cycle and one presented with mild thoracic pain assumed to be related to 5FU. Among the other patients, 8 developed alopecia and renal function worsened in one, leading to discontinuation of the treatment.

DISCUSSION

Despite the lack of proof of efficacy, doxorubicin is commonly used as a first line therapy for HCC. Recent randomized studies with doxorubicin[3,4] have confirmed the minimal efficacy of this drug in HCC. Results obtained in phase Ⅱ studies with different regimens using new cytotoxic drugs have not been very impressive. Thus, systemic chemotherapy cannot be considered as the standard of care for HCC patients. This situation could be related to a combination of poor efficacy and increased toxicity. Poor efficacy might result from intrinsic resistance caused by overexpression of multidrug resistance genes observed in most tumors. Obviously the underlying liver cirrhosis increases the risk of severe adverse events as many chemotherapeutic drugs are metabolized or eliminated via the liver. Moreover severe complications are certainly more likely if a cytotoxicity-related side effect occurs on a cirrhotic liver. Certain causes of the underlying cirrhosis, e.g. hepatitis B virus infection[5], may be reactivated after chemotherapy-induced immunodepression, producing an additive toxic effect.

In this particular retrospective series of HCC developed in normal or fibrotic livers, we attempted to estimate the raw efficacy and toxicity of systemic chemotherapy, regardless of liver status. The objective response rate in these patients given the ECF/ECC regimen was 22%, with a disease control rate (objective response plus stable disease) of 52%. The median time to progression was 6 mo, that is to say, quite similar to that observed using sorafenib[2], In addition, despite the fact that most tumors were huge, the reduction in tumor size was sufficient to allow surgical resection in 2 patients having only one huge tumor. Toxicity was mild and most side effects were manageable; one patient died suddenly between two courses. These two regimens (ECF and ECC) are very similar: capecitabine is the oral form of 5FU and, in a randomized 2 × 2 study conducted in advanced esophagogastric cancers[11], these two regimens were demonstrated to be effective. Such results with objective response rates approaching 20% have been reported in some other phase Ⅱ studies[12-14], including patients with or without cirrhosis: a similar ECC regimen gave, in a Korean series of 29 patients, an objective response rate of 24%[15]. Similar findings have been reported with the PIAF regimen (combination of CDDP, doxorubicin, 5FU and interferon)[16], where, in a series of 50 patients, 26% had a partial response including 9 who underwent surgical resection, and in 4, there were no viable cancer cells on resected specimens. Unfortunately, this did not translate into any difference in overall survival versus doxorubicin alone in a randomized phase Ⅲ trial despite the fact that less than half of the patients had underlying cirrhosis (but 17% of the patients had Child B class cirrhosis). The response rate of 22% we have observed in this series is a little bit higher than that observed in a retrospective series of 21 patients we have previously published[17] (16 having underlying cirrhosis). Comparing the results of these two series, we suggest that efficacy was better in the non-cirrhotic group. In our first series of 21 HCC patients we observed 3 responders (objective response rate of 14.5%)

including one non-cirrhotic patient (among cirrhotic patients, 2/16 were responders); median survival was 10 mo and surgery was possible in one patient. Toxicity appeared to be more frequent in the whole population, as we described 18 cases of grade 3-4 toxicities versus 7 cases in the current 24 non-cirrhotic patients. This might suggest that an ECF/ECC chemotherapy regimen (particularly the ECC regimen which is more convenient, especially in cirrhotic patients) could be delivered to non-cirrhotic patients with a hope of the possibility of secondary resection.

As a result of the recent SHARP trial, which demonstrated the efficacy of sorafenib in advanced-stage HCC, sorafenib is now the new standard of care in that setting. Combinations of sorafenib plus doxorubicin[18] seemed to be well tolerated and have yielded promising results in a randomized phase Ⅱ trial. A combination of sorafenib with the ECC regimen deserves a phase Ⅱ trial particularly in patients with HCC in a non-cirrhotic liver.

COMMENTS

Background

Systemic chemotherapy is not usually considered as a standard treatment in hepatocellular carcinoma (HCC) developed in cirrhotic patients.

Research frontiers

New targeted agents, like sorafenib, are efficient in advanced HCC and could be associated with systemic chemotherapy.

Innovations and breakthroughs

When systemic chemotherapy is given to patients who develop HCC without liver cirrhosis, the tolerance and efficacy are better and can allow curative surgery in some cases.

Applications

In selected cases of HCC without liver cirrhosis, systemic chemotherapy can be safely given with promising results.

Peer review

The peer reviewer emphasized the differences between these two regimens; one (ECF) requires a continuous infusion of chemotherapeutic drug (5FU) while the other is given orally and is easier to use.

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BRIEF ARTICLES

Early recognition of abdominal compartment syndrome in patients with acute pancreatitis

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Abstract

AIM: To assess the value of widely used clinical scores in the early identification of acute pancreatitis (AP) patients who are likely to suffer from intra-abdominal hypertension (IAH) and abdominal compartment syndrome (ACS).

METHODS: Patients (n = 44) with AP recruited in this study were divided into two groups (ACS and non-ACS) according to intra-abdominal pressure (IAP) determined by indirect measurement using the transvesical route *via* Foley bladder catheter. On admission and at regular intervals, the severity of the AP and presence of organ dysfunction were assessed utilizing different multifactorial prognostic systems: Glasgow-Imrie score, Acute Physiology and Chronic Health Evaluation Ⅱ (APACHE-Ⅱ) score, and Multiorgan Dysfunction Score (MODS). The diagnostic performance of scores predicting ACS development, cut-off values and specificity and sensitivity were established using receiver operating characteristic (ROC) curve analysis.

RESULTS: The incidence of ACS in our study population was 19.35%. IAP at admission in the ACS group was 22.0 (18.5-25.0) mmHg and 9.25 (3.0-12.4) mmHg in the non-ACS group (P < 0.01). Univariate statistical analysis revealed that patients in the ACS group had significantly higher multifactorial clinical scores (APACHE Ⅱ, Glasgow-Imrie and MODS) on admission and higher maximal scores during hospitalization (P < 0.01). ROC curve analysis revealed that APACHE Ⅱ, Glasgow-Imrie, and MODS are valuable tools for early prediction of ACS with high sensitivity and specificity, and that cut-off values are similar to those used for stratification of patients with severe acute pancreatitis (SAP).

CONCLUSION: IAH and ACS are rare findings in patients with mild AP. Based on the results of our study we recommend measuring the IAP in cases when patients present with SAP (APACHE Ⅱ > 7; MODS > 2 or Glasgow-Imrie score > 3).

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Key words: Acute pancreatitis; Abdominal compartment syndrome; Intra-abdominal pressure; Intra-abdominal hypertension; Organ dysfunction

Peer reviewer: Rakesh Kumar Tandon, Professor, Pushpawati Singhania Research Institute for Liver, Renal and Digestive Diseases, Sheikh Sarai Phase Ⅱ, New Delhi 110017, India

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INTRODUCTION

Acute pancreatitis (AP) remains a disease with an unpredictable clinical course, and significant associated morbidity and mortality[1]. Recently, the elevated intra-abdominal pressure (IAP) following the onset of AP has attracted growing attention, because it is increasingly recognized as an important risk factor for mortality in the early phase of the disease[2-4]. It was shown that intra-abdominal hypertension (IAH) is associated with higher mortality and morbidity rates, and prolonged ICU stay, in comparison to other patients who had normal IAP[5-8]. IAH has been recognized as a cause of organ dysfunction in critically ill patients, including those suffering from severe acute pancreatitis (SAP)[9-12]. Abdominal compartment syndrome (ACS) is defined as an increase of IAP > 20 mmHg, which is associated with occurrence of a new organ failure. A previously

reported incidence of ACS among patients with SAP ranges from 23% to 56%[11,13-15]. The mechanisms involved in the development of IAH and ACS include increased capillary permeability, hypoalbuminemia and volume overload, which produce a large retroperitoneal and visceral edema[6,16].

It has been shown that early recognition and treatment of IAH and ACS result in a significant improvement in patient survival and decreased morbidity. Due to its simplicity and minimal cost, the standard for intermittent IAP measurement is *via* the urinary bladder with a maximal instillation volume of 25 mL sterile saline[17]. Compared with bladder pressure measurements, clinical abdominal assessment showed poor sensitivity (56%) and accuracy (77%) for identifying elevated IAP[18]. It was shown that the essential approach to diagnosis and management of ACS is a timely IAP measurement. It is still not clear whether early IAP measurement should be routine for all AP patients and which patients would benefit most from the IAP monitoring.

This study aimed to assess the value of Acute Physiology and Chronic Health Evaluation Ⅱ (APACHE Ⅱ), Multiorgan Dysfunction Score (MODS) and Glasgow-Imrie clinical scores in early recognition of patients who are likely to suffer from IAH and ACS, and who would benefit from IAP monitoring and management. We also investigated the incidence of ACS, the role of its interventional management and clinical outcomes in patients with AP.

MATERIALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The Regional Ethics Committee approved the study (protocols no. BE-2-47 and P1-113/2005) and all patients provided written informed consent.

Study design and patient population

The study population included 44 patients with AP admitted to the Department of Surgery, Kaunas University of Medicine Hospital, from May 2007 to February 2008. General inclusion criteria were defined as follows: (1) a time interval between onset of typical abdominal symptoms and study inclusion of 72 h and less; (2) at least 3-fold elevated serum amylase or lipase levels; (3) no previous history of acute or chronic pancreatitis. On the first day of admission, the severity of the AP and presence of organ dysfunction were assessed utilizing three different multifactorial prognostic systems: Glasgow-Imrie score, APACHE-Ⅱ score, and MODS. Later, the severity of disease and clinical status were repeatedly reassessed using the same prognostic tools every 7 d, and when the deterioration of clinical condition occurred and after interventional treatment of ACS. The contrast-enhanced CT scan was performed on day 4 to 7 after the onset of disease to demonstrate the presence of pancreatic necrosis. According to the clinical course and clinical severity scores (APACHE Ⅱ > 7; Glasgow-Imrie > 2; MODS > 2; peak C-reactive protein value > 150 mg/L) patients were stratified into mild and severe AP groups. The data were prospectively recorded in a specially created database. All patients were treated according to our standard AP management protocol following the recent international guidelines.

Measurement of IAP and clinical assessment of patients

For IAP measurement, we used a standard two-way 16 Fr. Foley catheter inserted into the urinary bladder. The patient was placed in supine position. Twenty-five milliliters of 0.9% sterile NaCl were instilled and the catheter was connected to a tube from the urine collection bag. The pubic symphysis was considered level 0 and IAP was measured in cm H_2O, then recalculated in mmHg. IAP was measured every 24 h during a period of 3 d in all patients. For patients that developed IAH (IAP > 12 mmHg), the conservative treatment (according to the recommendations of international experts on IAH and ACS) was initiated and IAP was monitored every 12 h until the normal IAP was reached and sustained at least for 24 h. In cases when IAH > 18 mmHg was recorded, IAP was monitored every 4-6 h until IAP normalized or ACS developed. ACS was defined as an increase of IAP > 20 mmHg, which is associated with occurrence of a new organ failure[16,17].

Statistical analysis

Statistical analysis was performed using SPSS® for Windows release 16.0 (SPSS, Chicago, IL, USA). The quantitative variables are presented as mean ± SD or median (with interquartile range). For comparison between groups, the Mann-Whitney test, Student's *t* test or χ^2 test was employed where appropriate. The diagnostic performance of scores predicting ACS development, cut-off values and specificity and sensitivity of prognostic tools were established using receiver operating characteristic (ROC) curve analysis. Results with $P < 0.05$ were considered statistically significant.

RESULTS

A total of 44 patients with AP were included in the study. Demographic and clinical data of these patients are represented in Table 1.

All patients were divided into ACS and non-ACS groups. Median IAP in the ACS group at admission was 22.0 (18.5-25.0) mmHg and 9.25 (3.0-12.4) mmHg in the non-ACS group ($P < 0.01$) (Figure 1).

Differences of APACHE Ⅱ, Glasgow-Imrie and MODS median scores on admission and maximal scores (Max) during hospitalization period between ACS and non-ACS groups are presented in Table 2. The study revealed that patients in the ACS group had significantly higher multifactorial clinical scores on admission and higher maximal scores during hospitalization ($P < 0.01$). There were no significant differences between median admission and maximal scores of APACHE Ⅱ, Glasgow-Imrie and MODS within ACS or non-ACS groups ($P > 0.05$). The ACS group was characterized by

Table 1 Demographic and clinical variables of the whole series

Demographic and clinical variables	Values
Number of patents	44
Age (years ± SD)	49 ± 18
Gender male	70.4% (31/44)
SAP	70.4% (31/44)
Presence of necrosis	49.9% (18/44)
Extent (percentage) of necrosis	
< 30%	38.9% (7/18)
30%-50%	11.1% (2/18)
> 50%	50.0% (9/18)
IAH (IAP ⩾ 12 mmHg)	43.2% (19/44)
ACS (IAP ⩾ 20 mmHg + MOF)	13.6% (6/44)
Mortality	13.6% (6/44)
APACHE Ⅱ score at admission[1]	6.5 (4.0-10.0)
Max APACHE Ⅱ score during hospitalization[1]	8.0 (5.0-11.0)
Glasgow-Imrie score at admission[1]	3.0 (2.0-3.0)
Max Glasgow-Imrie score during hospitalization[1]	3.0 (2.0-4.0)
MODS score at admission[1]	2.0 (1.0-3.0)
Max MODS score during hospitalization[1]	2.0 (1.0-4.0)

[1]Values of clinical multifactorial scores is expressed as median (lower & upper quartiles).

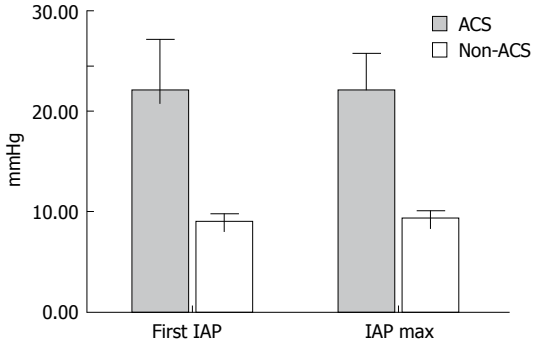

Figure 1 The 1st IAP and maximal IAP value in ACS and non-ACS groups. All patients were divided in ACS (grey colour) and non-ACS (white colour) groups. Median IAP at admission was 22.0 and 9.25 mmHg in ACS and non-ACS groups respectively ($P < 0.01$). There was no significant difference between value of the 1st measurement and maximum observed value of IAP within each group.

a markedly higher incidence of severe and necrotizing AP, and by the presence of high volume pancreatic necrosis in comparison to the non-ACS group. Mortality rate in the ACS group was also significantly higher, when compared to the non-ACS group (Table 3).

SAP was diagnosed in 70.4% (31/44) of all cases in this study group. We believe such a relatively high incidence of SAP is associated with the concentration of patients with severe disease in our tertiary care center referred from other regional hospitals, and a special focus on the patients with systemic inflammatory response syndrome and multiorgan failure (MOF). Nevertheless, the incidence of ACS in our study population was 19.35% (6/31) and did not exceed the prevalence of IAH and ACS shown in other clinical studies. Interestingly, the prevalence of IAH was significantly lower in the mild AP group, with only 7.69% (1/13) when compared to 58.06% (18/31) in the SAP group ($P > 0.01$). Specifically, all cases of ACS occurred in the SAP group with an incidence of 19.35% (6/31), while there were no cases of ACS in the mild AP group (0/13).

Table 2 Clinical scores (on admission and max value) in relation to presence of ACS

Clinical scores	ACS group median (lower & upper quartiles)	Non-ACS group median (lower & upper quartiles)	*P*
APACHE Ⅱ score on admission	12.0 (9.0-1.0]	6.0 (4.0-9.0)	< 0.01
Max APACHE score	14.0 (11.0-18.0)	7.0 (4.0-10.0)	< 0.01
Glasgow-Imrie score on admission	5.0 (4.0-5.0)	2.0 (2.0-3.0)	< 0.01
Max Glasgow-Imrie score	5.0 (4.0-5.0)	2.5 (1.0-3.0)	< 0.01
MODS score on admission	4.5 (3.0-8.0)	1.5 (1.0-3.0)	< 0.01
Max MODS score	4.5 (3.0-8.0)	2.0 (1.0-3.0)	< 0.01

Table 3 Clinical characteristics of patients with and without ACS

Clinical characteristics	ACS group (*n* = 6, %)	Non-ACS group (*n* = 38, %)	*P*
Severe AP	6 (100)	25 (65.8)	NS
Necrotizing AP	5 (83.3)	13 (34.2)	< 0.05
Necrosis > 30%	5 (83.3)	6 (15.8)	< 0.05
Deaths	4 (66.6)	2 (5.2)	< 0.01

Table 4 Areas under the ROC curves for prognostic factors of ACS

Variables	Area	Std. error	Asymptotic sig.	Confidence Interval	
				lower	upper
1st IAP on admission	0.932	0.065	0.001	0.805	1.059
Glasgow-Imrie score	0.921	0.054	0.001	0.816	1.026
APACHE Ⅱ score	0.866	0.062	0.004	0.745	0.987
MODS score	0.829	0.098	0.010	0.636	1.022

Table 5 Cut-off points, sensitivity and specificity for prognostic factors of ACS

Variable	Cut-off	Sensitivity (%)	Specificity (%)
APACHE Ⅱ score	> 7	100.0 (54.1-100.0)	60.5 (43.4-75.9)
Glasgow-Imrie score	> 3	83.3 (36.1-97.2)	86.8 (71.9-95.5)
MODS score	> 2	83.3 (36.1-97.2)	73.7 (56.9- 86.6)
1st IAP on admission	> 18	83.3 (36.1-97.2)	100.0 (90.7-100.0)

The diagnostic performance of multifactorial clinical scores and the first IAP measurement in predicting development of ACS during the course of AP were assessed using ROC curve analysis. ROC analysis revealed that all the analysed clinical scores are of good prognostic value in determining patients who are likely to further develop ACS (Figure 2). The areas under the ROC curves, cut-off values, specificity and sensitivity of prognostic tools are presented in Tables 4 and 5.

DISCUSSION

Our study confirmed the earlier published observations that AP is a risk factor for development of IAH and ACS[13,14,19,20]. Overall, somewhat higher rates of SAP in our institution could be explained by the concentration

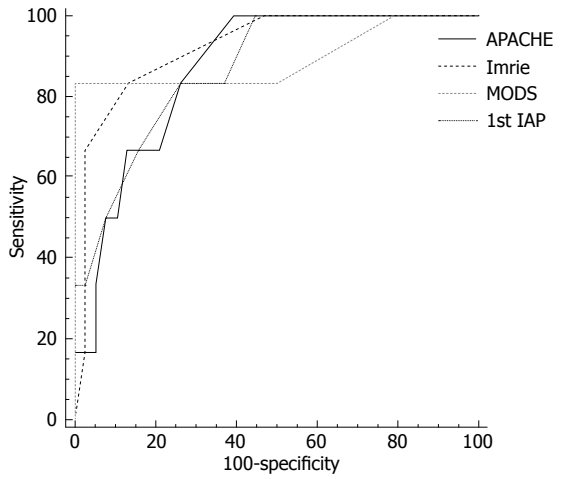

Figure 2 ROC curve analysis of prognostic factors for ACS development. ROC analysis revealed that clinical scores (APACHE Ⅱ, Glasgow-Imrie and MODS) and first IAP measurement on admission are good prognostic markers in determining patients who are likely to develop ACS. There was no significant difference between the areas under the ROC curves for these prognostic markers.

of the patients, because our hospital is a tertiary care center and many patients with suspected severe disease are referred to it from regional hospitals. However, the incidence of IAH and ACS in our study was similar to that observed in other studies, and as expected, it was associated with a higher incidence of MOF and higher mortality rates.

An early diagnosis of ACS and its adequate management is crucial[1,8,15,17]. The measurement and monitoring of IAP *via* urinary bladder catheter is a simple procedure, which requires virtually no technical skills and little resources. However, this procedure is invasive and is associated with significant discomfort for the patient[21,22]. It has also been shown that indwelling urinary catheters are associated with a higher incidence of infectious complications and prevalence of nosocomial pathogens[23-25]. Clearly, placement of a urinary catheter should not be routinely recommended for all patients, especially not for those who are unlikely to develop ACS. Therefore clinical assessment in selecting the patients that are likely to develop ACS is of particular importance. Our study demonstrated that development of IAH and ACS during the AP could be predicted by the use of clinical multifactorial scoring systems (APACHE Ⅱ, MODS, Glasgow-Imrie score), thus allowing a timely and appropriate selection of patients for this invasive procedure during the first hours and days of the disease. Clinical scores of patients who eventually suffered from IAH were higher during the first days in comparison to the group of patients with normal IAP. These findings are in accord with the observations of other groups[2,4,20,26]. The ROC analysis disclosed that APACHE Ⅱ, MODS, and Glasgow-Imrie scores have similar cut-off values to those used for the prediction of SAP. IAP > 18 mmHg on admission is also a valuable indicator that the patient has a higher risk for persistent IAH and development of ACS during the course of AP. All these prognostic markers had a good sensitivity, specificity and large area under the curve. Furthermore, the use of a clinical scoring system in combination with the first IAP measurement (eg. APACHE Ⅱ + first IAP on admission) allows us to identify nearly 100% of patients who are likely to develop SAP and suffer from ACS.

Previously published studies do not provide us with any useful recommendations or criteria for the selection of the AP patients for the IAP measurement and monitoring, although it would be unnecessary in the majority of cases when patients have a mild and self-limiting disease.

Based on the results of our study we recommend measuring the IAP only in cases when patients present with SAP (i.e. APACHE Ⅱ > 7; MODS > 2 or Glasgow-Imrie score > 3). We advocate a continuous monitoring of IAP in all cases when the patient suffers from SAP and has an IAP > 18 mmHg on first measurement. We would recommend utilizing the simpler Glasgow-Imrie or MODS scores in daily clinical practice and a more complex APACHE Ⅱ score in the clinical trial setting.

CONCLUSION

Placement of a urinary catheter for the monitoring of IAP would be unnecessary in the majority of AP cases, when patients have a mild and self-limiting disease.

We recommend measuring the IAP only in cases when patients present with SAP (i.e. APACHE Ⅱ > 7; MODS > 2 or Glasgow-Imrie score > 3). We advocate a continuous monitoring of IAP in all cases when the patient suffers from SAP and has an IAP > 18 mmHg on first measurement.

COMMENTS

Background

Acute pancreatitis (AP) remains a disease with an unpredictable clinical course, and significant associated morbidity and mortality. Recently, the elevated intra-abdominal pressure (IAP) after onset of AP has gained growing attention, because it is increasingly recognized as an important risk factor for mortality in the early phase of the disease. Intra-abdominal hypertension (IAH) has been recognized as a cause of organ dysfunction in critically ill patients, including those suffering from severe acute pancreatitis (SAP).

Research frontiers

It has been shown that early recognition and treatment of IAH and abdominal compartment syndrome (ACS) result in a significant improvement in patient survival and decreased morbidity, however, clinical abdominal assessment showed poor sensitivity and accuracy for identifying the elevated IAP. The essential approach to the diagnosis and management of ACS is a timely IAP measurement. It is still not clear whether early IAP measurement should be a routine for all AP patients and which patients would benefit most from the IAP monitoring.

Innovations and breakthroughs

An early diagnosis of ACS and its adequate management is crucial. The measurement and monitoring of IAP *via* urinary bladder catheter is a simple procedure, which requires virtually no technical skills and little resources. However, this procedure is invasive and is associated with significant discomfort for the patient. It has also been shown that indwelling urinary catheters are associated with a higher incidence of infectious complications and prevalence of nosocomial pathogens. Clearly, placement of a urinary catheter should not be routinely recommended for all patients, especially not for those who are unlikely to develop ACS. For the first time, our study demonstrated that development of IAH and ACS during the AP could be predicted by the use of clinical multifactorial scoring systems [Acute Physiology and Chronic Health

Evaluation Ⅱ (APACHE-Ⅱ), Multiorgan Dysfunction Score (MODS), Glasgow-Imrie score], thus allowing a timely and appropriate selection of patients for this invasive procedure during the first hours and days of the disease.

Applications

Based on the results of our study, we recommend measuring the IAP only in cases when patients present with SAP (i.e. APACHE Ⅱ > 7; MODS > 2 or Glasgow-Imrie score > 3). They advocate a continuous monitoring of IAP in all cases when the patient suffers from SAP and has an IAP > 18 mmHg on first measurement.

Terminology

ACS is a severe increase in the pressure within the abdomen (IAP) such that a patient's internal organs begin to fail and malfunction. This is a medical emergency. Untreated, ACS has a high mortality rate. There are a number of different methods that your doctor may use to treat the ACS. These may include giving medications to sedate or temporarily paralyze you or your loved one, placing tubes through the nose and into the stomach to remove fluid and air, placing tubes into the abdomen to remove fluid or blood, or opening the abdomen to release the increased pressure. Most patients with IAH and/or ACS will be cared for in an ICU where doctors and nurses constantly monitor for signs of illness and treat patients to keep their heart, lungs, kidneys, liver, and intestines functioning as normally as possible.

Peer review

An important aspect of AP has been addressed in this paper, as not much has been written about IAP and ACS in relation to AP. The study design is simple and clear.

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BRIEF ARTICLES

Outcome of laparoscopic cholecystectomy is not influenced by chronological age in the elderly

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Abstract

AIM: To evaluate the outcome of laparoscopic cholecystectomy (LC) in patients aged 80 years and older.

METHODS: A total of 353 patients aged 65 to 79 years (group 1) and 35 patients aged 80 years and older (group 2) underwent LC. Patients were further classified into two other groups: those with uncomplicated gallbladder disease (group A) or those with complicated gallbladder disease (group B).

RESULTS: There were no significant differences between the age groups (groups 1 and 2) with respect to clinical characteristics such as age, gender, comorbid disease, or disease presentation. Mean operative time, conversion rate, and the incidence of major postoperative complications were similar in groups 1 and 2. However, the percentage of high-risk patients was significantly higher in group 2 than in group 1 (20.0% *vs* 5.7%, $P < 0.01$). Group A comprised 322 patients with a mean age of 71.0 ± 5.3 years, and group B comprised 51 patients with a mean age of 69.9 ± 4.8 years. In group B, mean operative time (78.4 ± 49.3 min *vs* 58.3 ± 35.8 min, $P < 0.01$), mean postoperative hospital stay (7.9 ± 6.5 d *vs* 5.0 ± 3.7 d, $P < 0.01$), and the incidence of major postoperative complications (9.8% *vs* 3.1%, $P < 0.05$) were significantly greater than in group A. The conversion rate tended to be higher in group B, but this difference was not significant.

CONCLUSION: Perioperative outcomes in elderly patients who underwent LC seem to be influenced by the severity of gallbladder disease, and not by chronologic age. In octogenarians, LC should be performed at an earlier, uncomplicated stage of the disease whenever possible to improve perioperative outcomes.

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Key words: Elderly; Laparoscopic cholecystectomy; Octogenarians; Gallbladder; Cholecystitis

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INTRODUCTION

The Korean population is steadily aging. The percentage of the population 65 years of age and older was 5.8% in 1995 and was 9.1% in 2005. This age group is expected to grow from 14.3% in 2018 to 20.8% in 2026[1]. The prevalence of gallstone formation increases with age[2]. The reported incidence of cholelithiasis in the very elderly (80 years and older) is as high as 38%-53%[3,4]. This age group has a high incidence of complicated gallstone disease, such as acute cholecystitis, choledocholithiasis, and gallstone pancreatitis[5]. Laparoscopic cholecystectomy (LC) is currently the

procedure of choice for the management of gallbladder disease[6]. Many reports have demonstrated the relative safety and efficacy of LC, with low conversion rates and low postoperative morbidity compared to open cholecystectomy (OC)[7,8]. Currently, LC is being used with increasing frequency in elderly patients. Advanced age may be associated with increased postoperative complications and high conversion rates[9]. However, as life expectancy continues to increase, octogenarians are becoming a growing proportion of the population undergoing LC. Therefore, the purpose of this study was to evaluate the outcome of LC in patients aged 80 years and older compared to patients aged 65 to 79 years.

MATERIALS AND METHODS

Study group

A retrospective analysis of patients aged 65 years and older who underwent LC from November 1991 to January 2006 at Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul, Korea was performed. A total of 353 patients aged 65 to 79 years (group 1) and 35 patients aged 80 years and older (group 2) underwent LC. According to disease presentation, patients were further classified into two other groups: one with uncomplicated gallbladder disease (group A) and one with complicated gallbladder disease (group B). The diagnosis of gallbladder disease was based on a combination of clinical, laboratory, and radiologic findings. The most common imaging techniques used were ultrasonography and computed tomography. The diagnosis was confirmed by pathologic evaluation and surgical inspection. Whenever choledocholithiasis was suspected, preoperative endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography (MRCP) was performed. Endoscopic sphincterotomy with stone extraction was performed as needed. Since March 2004, percutaneous transhepatic gallbladder drainage (PTGBD) has been performed at our institution for patients admitted with suspected complicated acute cholecystitis (gallbladder empyema, gangrenous cholecystitis, perforated acute cholecystitis, emphysematous cholecystitis, and pericholecystic abscess).

Surgical technique

All LC procedures were carried out by one surgeon who had previous experience of more than 3000 laparoscopic cholecystectomies, assisted by a resident. A 10-mm Visiport® trocar (Tyco, Norwalk, USA) was inserted at the subumbilical area as the first port site. Two additional laparoscopic cannulae were inserted; one in the right upper quadrant (5 mm) and the other in the epigastric area (12 mm). A soft silastic drain was used in all patients.

Statistical analysis

The clinical characteristics, duration of LC surgery, conversion rate, complication rate, and postoperative hospital stay were analyzed using a statistical analysis program package (SPSS 15.0, SPSS Inc. Chicago, IL, USA). The results are expressed as mean ± SD. The statistical significance of observed differences was tested using the χ^2 test, Fisher's exact test, or Mann-Whitney test. A probability of 0.05 or less was $P < 0.05$ considered statistically significant.

Table 1 Preoperative clinical characteristics *n* (%)

	Group 1, 65-79 yr (*n* = 353)	Group 2, ≥ 80 yr (*n* = 35)	*P* value
Age	69.8 ± 3.7	82.9 ± 2.9	< 0.001
Male	152	13	0.499
Comorbid disease	166 (47.0)	18 (51.4)	0.619
Diabetes mellitus	67 (19.0)	11 (31.4)	0.080
Hypertension	111 (31.4)	6 (25.7)	0.484
Chronic liver disease	9 (2.5)	1 (2.9)	1.000
Ischemic heart disease	4 (1.1)	0 (0.0)	1.000
Cerebrovascular accident	2 (0.6)	0 (0.0)	1.000
Other	10 (2.8)	1 (2.9)	1.000
Diagnosis			
Uncomplicated gallbladder disease	294 (83.3)	28 (80.0)	0.622
Complicated gallbladder disease	47 (13.3)	4 (11.4)	1.000
Gallbladder cancer	12 (3.4)	3 (8.6)	0.144
Preoperative ERCP	33 (9.3)	4 (11.4)	0.761
CBD stone	29 (8.2)	4 (11.4)	0.522
Prior abdominal surgery	78 (22.1)	8 (22.9)	0.918
Preoperative PTGBD	12 (3.4)	1 (2.9)	1.000
ASA score			< 0.01
1 + 2	333 (94.3)	28 (80.0)	
3 + 4	20 (5.7)	7 (20.0)	

ERCP: Endoscopic retrograde cholangiopancreatography; CBD: Common bile duct; PTGBD: Percutaneous transhepatic gallbladder drainage; ASA: American society of anesthesiologists.

RESULTS

Overview of patients

There were no significant differences among the age groups (group 1 and 2) with respect to clinical characteristics such as age, gender, additional disease, and disease presentation. However, the percentage of high-risk patients was significantly higher in group 2 (20.0%) than in group 1 (5.7%). High risk was defined as an American Society of Anesthesiologists (ASA) score of 3 or 4 (Table 1).

Perioperative outcomes

Three hundred and twenty-two patients (group A) underwent LC for uncomplicated gallbladder disease (recurrent biliary colic, gallbladder polyp, and chronic cholecystitis), 51 patients (group B) underwent LC for complicated gallbladder disease (acute cholecystitis, gallbladder empyema, gangrenous cholecystitis, perforated acute cholecystitis, emphysematous cholecystitis, pericholecystic abscess, biliary pancreatitis, and cholangitis), and the remaining 15 patients underwent LC for gallbladder cancer. Not all gallbladder

Table 2 Perioperative outcome according to age group *n* (%)

	Group 1, 65-79 yr (*n* = 353)	Group 2, ≥ 80 yr (*n* = 35)	*P* value
Operative time (min)	60.8 ± 38.4	82.6 ± 47.6	0.087
Conversions	9 (2.5)	2 (5.7)	0.260
Complications	15 (4.2)	2 (5.7)	0.659
Bile leakage	6	1	
Intraabdominal fluid collection	3	1	
Wound infection	3	0	
Subumbilical wound hernia	1	0	
Bleeding	2	0	
Postoperative hospital stay (d)	5.5 ± 4.2	5.1 ± 4.0	0.873

cancers were diagnosed before surgery. One patient in group 1 had a pT2 tumor and underwent complete radical surgery of the gallbladder bed with lymph node dissection. Preoperative PTGBD was performed for 12 patients in group 1 and for one patient in group 2. The timing of LC in patients with acute cholecystitis was variable (early, delayed, or scheduled after PTGBD) according to clinical factors such as specialist or operating theater availability and the patient's medical condition.

Mean operative time and the conversion rate to OC were similar for the two age groups. The reasons for conversion were difficulty in gallbladder exposure or dissection at the Calot triangle because of dense adhesions. The incidence of major postoperative complications was similar in the two groups; 15 patients in group 1 (4.2%) and 2 patients in group 2 (5.7%). Six patients in group 1 and one patient in group 2 developed bile leakage after surgery. All patients were treated conservatively and the leakage subsided spontaneously within 7 d. Three patients in group 1 and one in group 2 had intraabdominal fluid collection; all patients were treated by ultrasound-guided percutaneous catheter drainage and parenteral antibiotics. Two patients in group 1 presented with bloody discharge from the subhepatic silastic drain on the first postoperative day, but the bleeding was controlled conservatively without blood transfusion. Postoperative wound infection occurred in three patients in group 1. One patient in group 1 developed a subumbilical wound hernia (10 mm cannula wound), which was surgically treated. The length of postoperative hospital stay was similar in the two groups (Table 2).

Group A (uncomplicated gallbladder disease) comprised 322 patients with a mean age of 71.0 ± 5.3 years, and group B (complicated gallbladder disease) comprised 51 patients with a mean age of 69.9 ± 4.8 years. In group B, the mean operative time was 78.4 ± 49.3 min, and this was significantly longer than that in group A (58.3 ± 35.8 min, $P < 0.01$). The incidence of major postoperative complications was significantly higher (9.8% *vs* 3.1%, $P < 0.05$) and mean postoperative hospital stay was significantly longer (7.9 ± 6.5 d *vs* 5.0 ± 3.7 d, $P < 0.01$) in group B compared to group A. The conversion rate tended to be higher in group B (5.9%) compared to group A (2.2%), but this difference was not significant ($P = 0.144$) (Table 3).

Table 3 Perioperative outcome according to disease presentation *n* (%)

	Group A (*n* = 322) Uncomplicated	Group B (*n* = 51) Complicated	*P* value
Age	71.0 ± 5.3	69.9 ± 4.8	0.125
Preoperative WBC count (/mm^3)	7048 ± 2166	11 928 ± 4269	< 0.001
Operative time (min)	58.3 ± 35.8	78.4 ± 49.3	< 0.01
Conversions	7 (2.2)	3 (5.9)	0.144
Complications	10 (3.1)	5 (9.8)	< 0.05
Postoperative hospital stay (d)	5.0 ± 3.7	7.9 ± 6.5	< 0.01

DISCUSSION

Gallbladder disease is the most common indication for abdominal surgery in the elderly[4,10,11]. In Korea, the age distribution of gallstones shows a peak incidence in the seventh decade and common bile duct stones show a peak incidence in the eighth decade[12]. Management of gallstones is important in the elderly because of a high rate of complicated biliary disease, increased postoperative morbidity, and prolonged hospital stay compared to younger patients[13,14]. However, recent reports have documented a mortality rate of 0% after LC in octogenarians[15,16]. In the present study, the mortality rate was 0% in octogenarians, with a 5.7% morbidity rate. This morbidity rate was comparable to that found in other studies (2.2-18.5%)[15-18]. Furthermore, there were no significant differences in operative time, conversion rate, complication rate, and postoperative hospital stay between the two age groups (group 1 *vs* group 2). The only difference observed was in the preoperative ASA score. Kwon *et al*[17] also found that there were no significant differences in the conversion rate, morbidity, mortality, and length of hospital stay between a group aged 65 to 79 years and a group aged ≥ 80 years. However, Pavlidis *et al*[16] reported that LC in the very elderly was associated with a higher conversion rate, increased morbidity, and longer hospital stay. Conversion to OC and postoperative complications may be associated with severe complicated gallbladder disease[19]. In the present study, operative time, postoperative complication rate, and postoperative hospital stay were also greater in patients who underwent LC for complicated gallbladder disease. In uncomplicated gallbladder disease, the overall conversion rate to OC was only 2.2% and the postoperative complication rate was also lower (3.1% *vs* 9.8%). These results may indicate that perioperative outcome is not influenced by chronologic age in the elderly, but is influenced by disease presentation.

When LC is performed in patients with severe cholecystitis, the rate of conversion to open surgery and postoperative complications is usually high. The

rate of conversion to open surgery in cases of severe cholecystitis is 8.7%-35%[20-23]. The complication rate associated with LC performed for acute cholecystitis ranges from 3% to 40%[19-22,24,25]. Therefore, since March 2004, we have adopted a protocol that includes PTGBD in patients admitted with suspected complicated acute cholecystitis (gallbladder empyema, gangrenous cholecystitis, perforated acute cholecystitis, emphysematous cholecystitis and pericholecystic abscess). Twelve patients in group 1 (3.4%) and one patient in group 2 (2.9%) underwent PTGBD before LC, and the conversion rate to OC was zero in both groups. Watanabe *et al*[26] reported that the combination of ultrasonography-guided PTGBD and LC was a safe and effective treatment for patients with acute suppurative cholecystitis. There were age no conversions to OC when LC was performed at a mean of 34.3 d after PTGBD. Moreover, a conversion rate of 3% was observed in patients who underwent surgery for acute cholecystitis 4 d after PTGBD[27]. In the present study, preoperative PTGBD may have had a positive effect on LC in complicated gallbladder disease.

Preoperative ERCP was performed in 33 patients in group 1 (9.3%) and 4 patients in group 2 (11.4%) who had clinical, laboratory, and radiological suspicion of choledocholithiasis, and 29 (87.9%) and 4 (100.0%), respectively, had common bile duct stones. All these patients underwent successful endoscopic sphincterotomy with stone extraction. The reported incidence of choledocholithiasis rises with age (26% and 43% in patients aged 65-79 years and 80-95 years, respectively)[10]. Therefore, preoperative ERCP in the elderly should be performed for patients with clinical, laboratory, and radiological suspicion of choledocholithiasis.

If elderly patients tend to have higher conversion and postoperative complication rates, and a longer postoperative hospital stay, this may be due to a higher incidence of complicated gallbladder disease[15]. In the present study, there was no difference in disease presentation between the two age groups; complicated gallbladder disease was 13.3% in group 1 and 11.4% in group 2. Therefore, despite higher ASA scores in group 2, perioperative outcomes might not be significantly different between the two groups.

In conclusion, perioperative outcomes in the elderly seem to be influenced by the severity of gallbladder disease, and not by chronologic age. In the very elderly, such as octogenarians, LC is also relatively safe, with acceptable morbidity compared to elderly patients younger than 80 years of age, if they have uncomplicated gallbladder disease. Therefore, in this age group, LC should be performed at an earlier, uncomplicated stage of the disease as often as possible to improve perioperative outcomes.

COMMENTS

Background

The Korean population is steadily aging. As life expectancy continues to increase, octogenarians are becoming a growing proportion of the population undergoing laparoscopic cholecystectomy (LC).

Research frontiers

Perioperative outcomes in the elderly seem to be influenced by the severity of gallbladder disease, and not by chronologic age. LC is relatively safe in the very elderly, such as octogenarians.

Related publications

Many reports have demonstrated the relative safety and efficacy of LC, with low conversion rates and low postoperative morbidity compared to open cholecystectomy. Currently, LC is being used with increasing frequency in elderly patients. Advanced age may be associated with increased postoperative complications and high conversion rates.

Innovations and breakthroughs

If elderly patients tend to have higher conversion and postoperative complication rates, and a longer postoperative hospital stay, this may be due to a higher incidence of complicated gallbladder disease.

Applications

In the very elderly, LC should be performed at an earlier, uncomplicated stage of the disease as often as possible to improve perioperative outcomes.

Peer review

The authors reported that outcome of LC is not influenced by chronologic age. This result is interesting.

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Usefulness of anti-ulcer drugs for the prevention and treatment of peptic ulcers induced by low doses of aspirin

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Abstract

AIM: To investigate the usefulness of anti-ulcer drugs for the prevention and treatment of low-dose aspirin-induced peptic ulcer.

METHODS: Upper gastrointestinal endoscopy was performed in 68 patients receiving daily low-dose aspirin (81 or 100 mg/day). The endoscopic findings were classified according to the Lanza score, and the scores were compared between groups categorized according to the concomitant use of anti-ulcer drugs and the types of drugs used. In another study, 31 hemorrhagic peptic ulcer patients who had been receiving low-dose aspirin were enrolled. The patients were randomly classified into the proton pump inhibitor (PPI)-treated group and the H2 receptor antagonist (H2RA)-treated group. The administration of low-dose aspirin was continued concomitantly, and endoscopic examinations were performed 8 wk later.

RESULTS: The Lanza scores (mean ± SD) of the gastro-mucosal lesions were 1.0 ± 1.9 and 1.9 ± 2.3 in 8 and 16 patients receiving prevention therapy with a PPI and an H2RA, respectively. Both scores were significantly smaller than the scores in 34 patients who were not receiving prevention therapy (4.7 ± 1.0) and in 10 patients receiving cytoprotective anti-ulcer drugs (4.3 ± 1.6). In the prospective study, 18 and 13 patients received a PPI and an H2RA, respectively. Endoscopic examinations revealed that the tissue in the region of the gastro-mucosal lesions had reverted to normal in all patients in the PPI-treated group and in 12 patients (92%) in the H2RA-treated group; no significant differences were observed between the groups.

CONCLUSION: H2RA therapy was effective for both the prevention and treatment of low-dose aspirin-induced peptic ulcer, similar to the effects of PPIs, while cytoprotective anti-ulcer drugs were ineffective in preventing ulceration.

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Key words: Hemorrhagic ulcer; H2 receptor Antagonist; Low-dose aspirin; Peptic ulcer; Proton pump inhibitor

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INTRODUCTION

Two major causes of peptic ulcers are infection with *Helicobacter pylori* (*H pylori*) and the administration of non-steroidal anti-inflammatory drugs (NSAIDs). Recently, much attention has been paid to NSAID-induced peptic ulcers, since the trend toward *H pylori* eradication using proton pump inhibitors (PPIs) and antibiotics is likely to reduce the incidence of *H pylori*-induced peptic ulcers in the future. Moreover, the increasing proportion of elderly individuals among the Japanese population is likely to produce a simultaneous increase in the prescription

of NSAIDs for the treatment of pain arising from osteoporosis and/or osteoarthritis. This trend may highlight the significance of NSAID-induced peptic ulcers. Low-dose aspirin has also been shown to induce peptic ulcers, similar to the effects of regular-dose aspirin and other NSAIDs[1]; this finding suggests that NSAID-induced peptic ulcers may become more common among the elderly population, since many patients receive antithrombotic therapy using low-dose aspirin for the treatment of cardiovascular diseases. Previously, we reported that NSAIDs were associated with hemorrhagic peptic ulcers in 28% of the patients seen between 2001 and 2004; the rate of patients receiving low-dose aspirin in this population was 27%, while the rates of patients receiving regular-dose aspirin, loxoprofen, diclofenac and other NSAIDs were 6%, 16%, 10% and 21%, respectively[1]. These data suggest that among the various NSAIDs in use, low-dose aspirin is the most important drug provoking peptic ulcers in Japan.

Infection with *H pylori* is frequently found in patients with NSAID-induced peptic ulcers, including those with low-dose aspirin-induced ulcers. According to our previous survey[1], a total of 82% of patients with hemorrhagic peptic ulcers tested positive for *H pylori* infection; the positivity rate was higher among those not treated with NSAIDs (88.6%) than among those receiving NSAIDs (67.2%). Of note, 62.5% of the patients with hemorrhagic peptic ulcers induced by low-dose aspirin tested positive for *H pylori*[1]. However, whether *H pylori* eradication prevents the development of peptic ulcers induced by low-dose aspirin remains controversial[2]. Chan *et al*[3] reported that both *H pylori* eradication and PPI administration were effective in preventing peptic ulcers induced by low-dose aspirin. On the other hand, Lai *et al*[4] revealed that hemorrhage recurred in more than 10% of the patients with low-dose aspirin-induced peptic ulcers, even after *H pylori* eradication, whereas PPI administration was effective in reducing the risk of recurrence. A therapeutic strategy to prevent peptic ulcers induced by NSAIDs, especially low-dose aspirin, is needed.

In the present study, we evaluated the efficacies of various anti-ulcer drugs (PPIs, H2RA and cytoprotective drugs) for the prevention and treatment of peptic ulcers induced by low-dose aspirin, by comparing the endoscopic findings of the patients to establish a therapeutic strategy for low-dose aspirin-induced peptic ulcers in Japanese patients.

MATERIALS AND METHODS

Study-1

Patients receiving daily low-dose aspirin (81 or 100 mg/day) and undergoing an upper gastrointestinal endoscopy at Saitama Medical University Hospital between February 2001 and September 2006 were enrolled in the study. Patients receiving NSAIDs other than aspirin and/or receiving regular-dose aspirin (from 1.0 to 4.5 g/day) were excluded from the analysis. Patients with malignancies or those who had undergone a gastrectomy were also excluded. The endoscopic findings were classified according to the Lanza score, as show in Table 1[5]. The scores were compared between groups categorized according to the concomitant use of anti-ulcer drugs and the types of drugs used.

Table 1 Classification of endoscopic findings according to the Lanza score[5]

Score	Endoscopic findings
0	No lesion
1	Hemorrhagic erosion
2	One or two erosions
3	3-10 erosions
4	More than 10 erosions
5	Ulcer

Study-2

The subjects comprised hemorrhagic peptic ulcer patients who had been receiving low-dose aspirin daily and who had been admitted to Saitama Medical University Hospital between February 2001 and March 2008. The exclusion criteria were similar to those used in Study-1: patients receiving NSAIDs other than aspirin and/or regular-dose aspirin and those with malignancies or who had undergone a gastrectomy at enrolment. Informed consent was obtained from all patients, and the subjects were randomly classified into two groups. The patients classified as the PPI-treated group were given either lansoprazole (15 or 30 mg, daily), rabeprazole sodium (10, 20 or 40 mg, daily) or omeprazole (20 mg, daily). In contrast, the patients in the H2RA-treated group were given famotidine (40 mg, daily). In all patients, the administration of low-dose aspirin was continued concomitantly with the PPI or famotidine therapy. Infection with *H pylori* was determined using a serum antibody test (AP-960; Scimed Life Systems/Boston Scientific Corp, Natick, MA, USA) or a culture of gastric mucosal specimens obtained during an endoscopic examination. *H pylori* eradication with the administration of lansoprazole (60 mg, daily), amoxicillin hydrate (1500 mg, daily) and clarithromycin (800 mg, daily) was performed for 7 d in patients with a positive infection status within 2 wk of the occurrence of peptic ulcer hemorrhage. The therapeutic efficacy of *H pylori* eradication was assessed by the urea breath test 8 wk later. An upper gastrointestinal endoscopy was performed 8 wk after the initiation of PPI or famotidine therapy. The ulcer was diagnosed as healed once scar formation at the site of the lesion was complete.

Statistical Analysis

The Mann-Whitney *U* test and the Fisher's exact test were used to analyze the data. Statistical significance was defined as $P < 0.05$.

RESULTS

Study-1

Sixty-eight patients (45 men and 23 women) between

Table 2 Anti-ulcer drugs used in patients enrolled in study-1

Type of drug	Drug name	Doses (/day)	Number of patients
None			34
PPIs			8
	Rabeprazole sodium	10 mg	3
	Lansoprazole	30 mg	2
	Lansoprazole	15 mg	1
	Omeprazole	20 mg	2
H2RAs			16
	Famotidine	40 mg	1
	Famotidine	20 mg	8
	Famotidine	10 mg	1
	Lafutidine	20 mg	2
	Nizatidine	300 mg	2
	Ranitidine hydrochloride	75 mg	1
	Cimetidine	100 mg	1
Cytoprotective anti-ulcer drugs			10
	Rebamipide	300 mg	2
	Rebamipide	200 mg	1
	Rebamipide	100 mg	1
	Azulensulfonate sidium + L-Glutamine	2 g	2
	Teprenone	1.5 g	1
	Polaprezinc	150 mg	1
	Sofalcone	300 mg	1
	Alginate sodium	180 mL	1

the ages of 25 and 88 years were enrolled in the study. Thirty-four patients received no anti-ulcer drugs, while 8, 16 and 10 patients were given PPIs, H2RAs and cytoprotective anti-ulcer drugs, respectively. The types and doses of the anti-ulcer drugs are shown in Table 2. Duration of daily low-dose aspirin therapy ranged from 1 to 3650 d, however, therapy duration (days; mean ± SD) did not differ in patients receiving and not receiving anti-ulcer drugs (898 ± 1384 and 1165 ± 1389, respectively). In the case of patients receiving anti-ulcer drugs, the prevention therapies were initiated simultaneously with low-dose aspirin administration. Anti-platelet and anticoagulant drugs were used in 5 and 1 patients receiving and not receiving anti-ulcer drugs, respectively. No differences in age or sex were observed among the patients not treated with anti-ulcer drugs and those receiving PPI, H2RA or cytoprotective anti-ulcer drugs (Table 3). However, the endoscopic scores were significantly smaller in patients receiving PPIs and H2RAs, compared with those receiving cytoprotective anti-ulcer drugs and those not receiving anti-ulcer drugs. A grade 5 endoscopic score was observed in 31 of 34 patients (91%) not receiving anti-ulcer drugs and in 8 of 10 patients (80%) receiving cytoprotective anti-ulcer drugs, while the grades ranged between 0 and 3 in 11 of 16 patients (69%) receiving H2RAs and in 7 of 8 patients (88%) receiving PPIs. The scores in patients receiving PPIs and in those receiving H2RAs were statistically similar. In addition, no differences were observed between the scores in 5 patients receiving regular-dose H2RAs and in 11 patients receiving low-dose H2RAs. Moreover, the scores in patients receiving cytoprotective anti-ulcer drugs and in those not receiving anti-ulcer drugs were similar. One 72-year-old male patient had an endoscopic score of grade 5 despite the use of a PPI. *H pylori* infection was not detected in this patient, but a coronary artery bypass grafting procedure had been performed 39 d prior to the endoscopic examination, and the patient had been receiving daily doses of warfarin in addition to low-dose aspirin since that time.

Study-2

Forty patients were enrolled in the study: 20 patients in the PPI-treatment group and 20 patients in the H2RA-treatment group. An endoscopic examination was performed at 8 wk in 18 patients (13 men and 5 women) aged 69.2 ± 13.4 years (mean ± SD) in the PPI-treatment group and in 13 patients (10 men and 3 women) aged 69.7 ± 9.5 years in the H2RA-treatment group; the 9 remaining patients were transferred to other hospitals prior to endoscopic examination. A positive *H pylori* infection status was seen in 12 and 10 patients (67% and 77%) in the PPI-treatment and H2RA-treatment groups, respectively; *H pylori* infection rate was similar in both groups. *H pylori* eradication was achieved in all patients who had a positive *H pylori* infection status. Endoscopic examinations performed at 8 wk revealed that the peptic ulcers had completely healed in 18 patients (100%) in the PPI-treatment group and in 12 patients (92%) in the H2RA-treatment group; no difference in therapeutic efficacy was seen between the two groups. The peptic ulcer in one patient with a positive *H pylori* infection status in the H2RA-treatment group had not completely healed at 8 wk. This patient had repeatedly received loxoprofen for the treatment of a headache 1 wk prior to the endoscopic examination, suggesting that peptic ulcers might recur in the presence of loxoprofen administration, even if the low-dose aspirin-induced ulcer had healed during famotidine therapy. There were 3 and 6 patients with a negative *H pylori* infection status in the H2RA-treatment and PPI-treatment groups, respectively. Peptic ulcers were completely healed in all these patients.

DISCUSSION

In the US and Europe, drugs such as PPIs[6], prostaglandins[7] and regular-dose H2RAs[8] have been reported to be effective in the prevention of NSAID-induced peptic ulcers, and PPIs and prostaglandins have been reported to effectively attenuate such ulcers[6]. In addition, studies conducted in Hong Kong and the US have revealed that both *H pylori* eradication[3] and PPI treatment[4] are useful in preventing the recurrence of hemorrhagic peptic ulcers induced by low-dose aspirin. In Japan, however, the administration of PPIs, H2RAs and prostaglandins for the prevention of peptic ulcers induced by NSAIDs and low-dose aspirin is not covered by medical insurance, although the use of these drugs for the treatment of peptic ulcers is covered. Among these drugs, PPIs and prostaglandins are widely accepted as the most effective therapeutic agents for NSAID-induced peptic ulcers, however, prostaglandins are seldom used due to their adverse effects such as abdominal pain and diarrhea. Thus, in Japan, many

Table 3 Endoscopic findings classified according to the Lanza score in patients receiving low-dose aspirin with or without anti-ulcer drugs

Number of patients	Sex M:F	Age (yr) (mean ± SD)	Lanza scores (Number of patients)						Mean scores
			0	1	2	3	4	5	
No anti-ulcer drugs									
34	24:10	69.4 ± 11.2	1	0	1	0	1	31	4.74[1]
Cytoprotective drugs									
10	8:2	67.9 ± 8.6	1	0	0	1	0	8	4.30[1]
H2RAs									
16	7:9	67.8 ± 14.0	7	3	1	0	0	5	1.88[1]
At regular doses									
5	2:3	69.6 ± 10.8	2	1	1	0	0	1	1.60[1]
Less than regular doses									
11	5:6	66.9 ± 15.6	5	2	0	0	0	4	2.00[1]
PPIs									
8	6:2	62.0 ± 17.0	6	0	0	1	0	1	1.00[1]

[1]$P < 0.05$ *vs* both no anti-ulcer drugs and cytoprotective drugs, according to the Mann-Whitney *U* test.

physicians usually use PPIs for the treatment of peptic ulcers induced by NSAIDs and low-dose aspirin. For the prevention of peptic ulcers, cytoprotective anti-ulcer drugs other than prostaglandins are commonly used, although no evidence supporting this use has been reported in the medical literature. Therefore, a therapeutic strategy for the treatment and prevention of peptic ulcers induced by NSAIDs, especially low-dose aspirin, should be established for Japanese patients.

Thus, we performed a retrospective study (Study-1) to clarify the efficacy of various anti-ulcer drugs to prevent peptic ulcers induced by low-dose aspirin and a prospective study (Study-2) to compare the therapeutic efficacies of PPIs and H2RAs for treating such ulcers. The retrospective study revealed that H2RAs effectively prevented peptic ulcers induced by low-dose aspirin to a degree similar to that of PPIs, however, cytoprotective drugs were not effective in preventing peptic ulcers. To our surprise, H2RA administration at doses less than the regular dose was also effective in preventing low-dose aspirin-induced peptic ulcers. Furthermore, the prospective study demonstrated that the therapeutic efficacies of PPIs and H2RAs at regular doses were almost equivalent. The secretion of gastric acids from the gastric mucosa has been shown to be lower in Japanese patients than in European and American patients[9,10], since marked atrophy of the gastric mucosa is often found in many Japanese patients due to the prevalence of *H pylori* infection. Thus, low-dose H2RAs, such as famotidine 20 mg daily, seem to be effective in the prevention of peptic ulcers induced by low-dose aspirin, and regular-dose H2RAs, such as famotidine 40 mg, may be sufficient to treat such peptic ulcers in Japanese patients. However, it should be noted that grade-5 endoscopic findings were observed in a 72-year-old patient in Study-1 receiving both warfarin and low-dose aspirin despite concomitant prevention therapy with a PPI. In addition, peptic ulcer healing did not occur in one patient receiving loxoprofen as well as low-dose aspirin in the H2RA-treated group in Study-2. The usefulness of H2RAs for the treatment and prevention of low-dose aspirin-induced peptic ulcers should be further investigated, focusing on elderly patients and those receiving warfarin as well as NSAIDs[11]. Moreover, the therapeutic and prevention efficacy of H2RA should be studied in relation to *H pylori* infection status, therapeutic effect of *H pylori* infection, history of upper gastrointestinal diseases and the types of peptic ulcer such as acute and chronic disorders in future research.

In conclusion, H2RA therapy was effective for both the prevention and treatment of low-dose aspirin-induced peptic ulcers, similar to the effects of PPIs, while cytoprotective anti-ulcer drugs were ineffective in preventing peptic ulcers. Considering the cost and adverse effects of PPIs and prostaglandins, H2RA may be the most beneficial anti-ulcer drug for the prevention and treatment of peptic ulcers induced by low-dose aspirin in Japan.

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COMMENTS

Background

The incidence of low-dose aspirin-induced peptic ulcer seems to be increasing in Japan in conjunction with the increasing proportion of elderly individuals, in whom metabolic syndrome frequently develops. However, a therapeutic and prevention strategy for such peptic ulcers has not yet been established.

Research frontiers

The effect of *Helicobacter pylori* (*H pylori*) eradication in the prevention of peptic ulcers induced by low-dose aspirin remains controversial. Chan *et al*[3] reported that both *H pylori* eradication and proton pump inhibitor (PPI) administration were effective in preventing peptic ulcers induced by low-dose aspirin. On the other hand, Lai *et al*[4] revealed that hemorrhage recurred in more than 10% of patients with low-dose aspirin-induced peptic ulcers, even after *H pylori* eradication, whereas PPI administration was effective in reducing the risk of recurrence.

Innovations and breakthroughs

H2 receptor antagonist (H2RA) was effective in both the prevention and treatment of low-dose aspirin-induced peptic ulcers, similar to the effects of

PPIs, while cytoprotective anti-ulcer drugs were ineffective in preventing ulcers.

Applications

Considering the cost and adverse effects of PPIs and prostaglandins, H2RAs may be the most beneficial anti-ulcer drugs for the prevention and treatment of peptic ulcers induced by low-dose aspirin in Japan.

Terminology

Low-dose aspirin: aspirin at regular doses (from 1.0 to 4.5 g/day) is administered to patients with fever, headache and arthralgia. In contrast, daily low-dose aspirin (81 or 100 mg/day) is used as antithrombotic therapy for patients with cardiovascular diseases.

Peer review

The incidence of low-dose aspirin-induced peptic ulcers is increasing in Japan, but the evidence is still lacking. In this paper, the authors evaluated the efficacies of various anti-ulcer drugs for the prevention and treatment of low-dose aspirin-induced peptic ulcers. The author concluded that the efficacies of H2RAs and PPIs for the prevention and treatment of L-Asp-induced peptic ulcers.

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BRIEF ARTICLES

Establishment of an animal model of ischemic type intrahepatic biliary lesion in rabbits

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Abstract

AIM: To explore a method to establish an animal model of ischemic type intrahepatic biliary lesion in rabbits.

METHODS: Forty Japanese white rabbits of clean grade were divided randomly into four groups (10 rabbits per group) including sham operation (SO) group, and artery-bile obstruction (ABO)-1 h group, ABO-2 h group and ABO-3 h group. All the rabbits in this study underwent the same initial surgical procedure in which the liver was prepared as for graft removal during liver transplantation. Subsequently in the SO group, no additional vascular intervention was performed, while in groups ABO-1 h, ABO-2 h and ABO-3 h, the animals underwent combined clamping of the hepatic artery and common bile duct with microvascular clips for 1, 2 and 3 h, respectively. After the scheduled occlusion time, the clip was removed to recover blood supply. The animals were killed 4 wk after operation. The survival rate, liver function, cholangiography and histopathological manifestation of the rabbits in each group were observed.

RESULTS: The survival rate was 100% in groups SO, ABO-1 h and ABO-2 h, while it was 60% in group ABO-3 h. At each observation time, the change degree of the indexes of liver function was proportional to the clamping time (ABO-3 h > ABO-2 h > ABO-1 h > SO, $P < 0.05$). Cholangiographical and histopathologic manifestations both showed that intrahepatic biliary lesion aggravated proportionally with the increase of the clamping time.

CONCLUSION: An animal model of ischemic type intrahepatic biliary lesion in rabbits is successfully established, which may provide a reliable technique for basic and clinical research into the etiology, development and prophylaxis of ischemic type intrahepatic biliary lesion after liver transplantation.

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Key words: Biliary complication; Ischemic type biliary lesion; Animal model; Liver transplantation; Intrahepatic biliary stricture; Ischemic reperfusion injury

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INTRODUCTION

Biliary complications, which occur at a rate of approximately 6%-35%[1], have long been recognized as a major cause of morbidity and graft failure in patients after orthotopic liver transplantation (OLT). The most troublesome is the so-called ischemic type biliary lesion (ITBL), with an incidence varying between 5% and 15%[2]. ITBL is a special biliary complication with non-anastomotic biliary tree destruction, which is one of the most important reasons for liver re-transplantation. For intrahepatic ITBL, especially in the presence of extensive intrahepatic ITBL, endoscopic and radiological techniques and surgical approaches are usually unsuccessful and re-transplantation is mostly unavoidable[2]. Therefore, it is urgent to establish an animal model of ischemic type intrahepatic biliary lesion to study the etiology, development and prophylaxis

of ITBL. In the present study, by combined clamping of the common bile duct and hepatic artery for 2 h, producing the biliary ischemia reperfusion injury, and raising the animals for 4 wk, an animal model of ischemic type intrahepatic biliary lesion in rabbits was successfully established.

MATERIALS AND METHODS

Animals and groups

Animal care and experimental procedures were carried out strictly in accordance with the guide for the care and use of laboratory animals (National Research Council of USA, 1996) and the related ethical regulations of our university. Forty Japanese white rabbits of clean grade, weighing 2.0-2.5 kg, were selected (provided by Institute of Laboratory Animal Science), irrespective of male or female gender. All of the rabbits were raised under the same condition including a temperature of 18-23℃, the relative humidity of 50%-60%, 12 h diurnal rhythm and freedom to eat and drink. The rabbits were divided randomly into four groups (10 rabbits per group) including sham operation (SO) group, and artery-bile obstruction (ABO)-1 h group, ABO-2 h group and ABO-3 h group.

Establishment of an animal model

The rabbits were prohibited to eat for 12 h and drink for 6 h before operation. They were anesthetized by injecting 1% pentobarbital (1-2 mL/kg) intravenously. The skin was prepared and disinfected routinely. Then, a median incision of the epigastrium about 6 cm in length was formed. All the rabbits in this study underwent the same initial surgical procedure in which the liver was prepared as for graft removal during liver transplantation. Upon the completion of this procedure, the liver was isolated from all vascular supply except for the main hepatic artery, the extra-hepatic peribiliary plexus and the portal vein. In the SO group, no additional vascular intervention was performed. In groups ABO-1 h, ABO-2 h and ABO-3 h, the animals underwent combined clamping of the hepatic artery and common bile duct with microvascular clips for 1, 2 and 3 h, respectively. After the scheduled occlusion time, the clip was removed to recover blood supply. All the animals from these groups were killed 4 wk after operation.

Observation indexes

Survival rate: The animals in each group were raised for 4 wk after operation, and the survival rates of each group were observed.

Examination of liver function: biochemical liver tests were monitored at different time points up to 4 wk including preoperation, and the 1 d, the 1, 2, 3 and 4 wk post-operation. Serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), total bilirubin (TBIL) and direct bilirubin (DBIL) were measured, using standard analytical methods.

Cholangiography: Four weeks after operation, all the rabbits in the four groups underwent cholangiography. Under routine anesthesia, the abdominal cavity was opened according to the former incision. Three to five milliliters of 30% meglucamine diatrizoate solution was injected slowly through the distal end of the common bile duct, and a X-ray photograph was taken to observe the intrahepatic biliary lesion.

Histopathologic examination: After cholangiography, the animals were killed to collect liver tissue samples at the hepatic hilum for histopathological examination. Serial 4-μm-thick sections of formalin-fixed, paraffin-embedded liver tissues were stained with hematoxylin-eosin.

Statistical analysis

Quantitative data were shown as mean ± SD. SPSS statistical software 13.0 was used to conduct analysis of variance of multiple means. For all analyses, a P value less than 0.05 was considered statistically significant.

RESULTS

Survival rates

The animals in groups SO, ABO-1 h and ABO-2 h lived throughout the period of the experiment with a survival rate of 100%. However, four rabbits in group ABO-3 h died on the days 5, 7, 12 and 14 after operation, respectively, with a survival rate of 60%. The causes of death included hepatic ischemic necrosis, bile leakage and abdominal infection, which was supported by autopsy and histopathological examination.

Examination of liver function

All the biochemical indexes (AST, ALT, ALP, GGT, TBIL and DBIL) in groups ABO-1 h, ABO-2 h and ABO-3 h increased with different degrees after operation. No biochemical abnormality was observed in group SO during the entire follow-up. The indexes reached a peak on the day 1 after operation, and then decreased gradually. At the end of the observation period (4 wk after operation), all biochemical abnormalities were spontaneously resolved except for the ALP and GGT in group ABO-2 h and ABO-3 h, which were still higher than that in group SO. At each observation time, the change in the indexes was proportional to clamping time (ABO-3 h > ABO-2 h > ABO-1 h > SO, $P < 0.05$) (Table 1).

Cholangiography

Four weeks after operation, all the rabbits in the four groups underwent cholangiography. The images of the intra-hepatic bile duct indicated that it was normal and no intrahepatic biliary lesion was visualized in groups of SO and ABO-1 h. However, a biliary lesion was observed obviously in groups ABO-2 h and ABO-3 h. It showed that intrahepatic biliary lesion aggravated proportionally with the increase in the clamping time (Figure 1).

Table 1 The change of biochemical indexes of rabbits in all groups (mean ± SD)

Groups	Preoperation	1 d post-operation	1 wk post-operation	2 wk post-operation	3 wk post-operation	4 wk post-operation
AST (U/L)						
SO	38.3 ± 2.4	38.1 ± 2.6	38.0 ± 2.4	38.7 ± 2.3	38.5 ± 2.1	39.1 ± 3.3
ABO-1 h	38.2 ± 2.3	260.3 ± 19.5[1]	85.0 ± 11.1[1]	39.0 ± 1.6	38.2 ± 2.5	39.0 ± 3.3
ABO-2 h	40.1 ± 1.7	359.9 ± 11.9[2]	154.8 ± 14.4[2]	86.9 ± 5.7[2]	39.1 ± 1.7	38.8 ± 1.9
ABO-3 h	39.8 ± 4.6	461.9 ± 11.4[3]	199.2 ± 16.9[3]	112.0 ± 16.7[3]	82.7 ± 9.7[3]	40.1 ± 3.6
ALT (U/L)						
SO	42.2 ± 2.2	42.0 ± 2.3	43.2 ± 2.0	40.0 ± 3.7	40.7 ± 3.4	42.0 ± 2.3
ABO-1 h	42.0 ± 2.4	200.5 ± 13.0[1]	169.8 ± 14.3[1]	65.9 ± 11.5[1]	42.3 ± 2.8	43.2 ± 2.0
ABO-2 h	43.9 ± 2.3	400.1 ± 12.3[2]	200.2 ± 12.0[2]	92.2 ± 8.6[2]	42.7 ± 3.1	42.2 ± 3.7
ABO-3 h	43.8 ± 4.6	505.9 ± 14.0[3]	292.0 ± 15.8[3]	116.6 ± 9.0[3]	87.7 ± 8.8[3]	44.6 ± 2.5
ALP (U/L)						
SO	117.0 ± 14.7	115.9 ± 15.9	114.3 ± 14.8	110.3 ± 9.6	113.3 ± 12.7	111.4 ± 11.7
ABO-1 h	117.6 ± 14.5	203.9 ± 18.8[1]	139.8 ± 10.3[1]	119.6 ± 9.0[1]	112.5 ± 9.3	109.5 ± 7.5
ABO-2 h	118.8 ± 14.2	304.8 ± 16.8[2]	173.1 ± 11.2[2]	136.2 ± 6.6[2]	138.8 ± 6.3[2]	139.6 ± 7.4[2]
ABO-3 h	113.9 ± 11.7	440.0 ± 29.4[3]	226.0 ± 13.7[3]	183.7 ± 9.8[3]	149.4 ± 10.2[3]	149.7 ± 11.5[3]
GGT (U/L)						
SO	16.7 ± 2.1	17.5 ± 2.5	18.0 ± 2.1	18.5 ± 2.6	18.8 ± 3.9	17.3 ± 2.3
ABO-1 h	18.7 ± 2.2	62.8 ± 10.2[1]	20.3 ± 3.4	17.3 ± 2.8	19.7 ± 3.5	17.5 ± 2.5
ABO-2 h	17.7 ± 2.8	111.5 ± 9.2[2]	106.5 ± 11.3[2]	81.9 ± 7.6[2]	84.7 ± 9.4[2]	59.6 ± 13.6[2]
ABO-3 h	17.9 ± 3.0	214.6 ± 18.6[3]	182.5 ± 8.6[3]	153.7 ± 14.4[3]	155.9 ± 19.3[3]	98.0 ± 6.8[3]
TBIL (μmol/L)						
SO	10.0 ± 2.0	9.5 ± 1.7	10.4 ± 2.0	10.1 ± 1.9	10.6 ± 2.2	10.8 ± 2.3
ABO-1 h	10.1 ± 1.8	21.0 ± 3.1[1]	10.6 ± 2.4	9.8 ± 1.6	9.5 ± 2.0	10.5 ± 2.3
ABO-2 h	9.9 ± 2.2	30.7 ± 2.3[2]	19.8 ± 1.9[2]	10.5 ± 2.2	9.3 ± 1.5	9.9 ± 2.0
ABO-3 h	9 .7 ± 1.9	40.7 ± 4.3[3]	30.6 ± 2.4[3]	18.1 ± 2.2[3]	10.3 ± 1.8	11.2 ± 2.3
DBIL (μmol/L)						
SO	3.9 ± 1.0	3.8 ± 1.5	3.9 ± 1.3	4.9 ± 1.6	4.5 ± 1.1	5.5 ± 1.2
ABO-1 h	4.7 ± 1.3	13.5 ± 2.5[1]	4.2 ± 1.3	4.8 ± 0.8	4.1 ± 1.2	4.6 ± 0.8
ABO-2 h	4.3 ± 1.3	20.4 ± 2.8[2]	13.4 ± 2.2[2]	4.9 ± 1.1	3.9 ± 1.1	4.6 ± 1.3
ABO-3 h	4.5 ± 1.1	33.7 ± 3.0[3]	19.6 ± 1.7[3]	11.2 ± 2.0[3]	5.0 ± 1.4	4.8 ± 1.5

[1]ABO-1 h *vs* SO, $P < 0.05$; [2]ABO-2 h *vs* ABO-1 h, $P < 0.05$; [3]ABO-3 h *vs* ABO-2 h, $P < 0.05$.

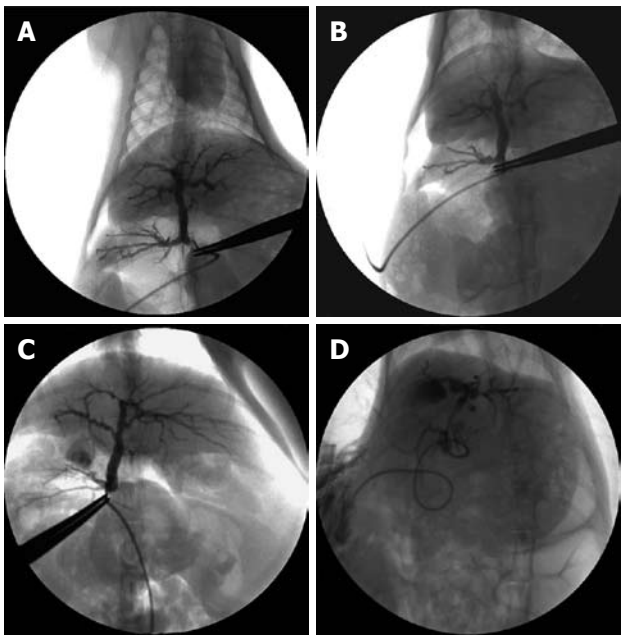

Figure 1 Cholangiography of the four groups 4 wk after operation. A (SO): The image of the intra-hepatic bile duct was normal. No biliary stricture or dilation was observed; B (ABO-1 h): The image of the intrahepatic bile duct resembled that of SO, no intrahepatic biliary lesion was visualized either; C (ABO-2 h): All the branches of the intrahepatic bile duct appeared loose, with the "string beads manifestation" in part of them; D (ABO-3 h): The image of the intrahepatic bile duct was obviously abnormal, with a loose appearance and severe damage.

Histopathological examination

After cholangiography, the sample of liver tissues at the hepatic hilum was taken for histopathological examination. The results indicated that the morphology of the intrahepatic bile duct epithelial cells was normal and there was no cell necrosis in groups SO and ABO-1 h. However, epithelial cells were obviously damaged and sloughed into the bile duct lumen in groups ABO-2 h and ABO-3 h. It also showed that the intrahepatic biliary lesion was aggravated proportionally with the clamping time (Figure 2).

DISCUSSION

ITBL is defined as non-anastomotic destruction of the graft's biliary tree after OLT and characterized by the formation of sludge or stone, bile duct destruction, and even non-function of the allograft[3,4]. A classification of ITBL has been proposed based on the localization of the abnormalities, distinguishing type I (extrahepatic lesions), type Ⅱ (intrahepatic lesions) and type Ⅲ (intra- and extrahepatic alterations)[1]. For type Ⅱ and type Ⅲ, especially for multiple and diffuse ITBL, there is poor prognosis and graft survival, with the result of liver function failure and inevitable re-transplantation in most patients. Therefore, it is of clinical significance to establish an animal model of ischemic type intrahepatic

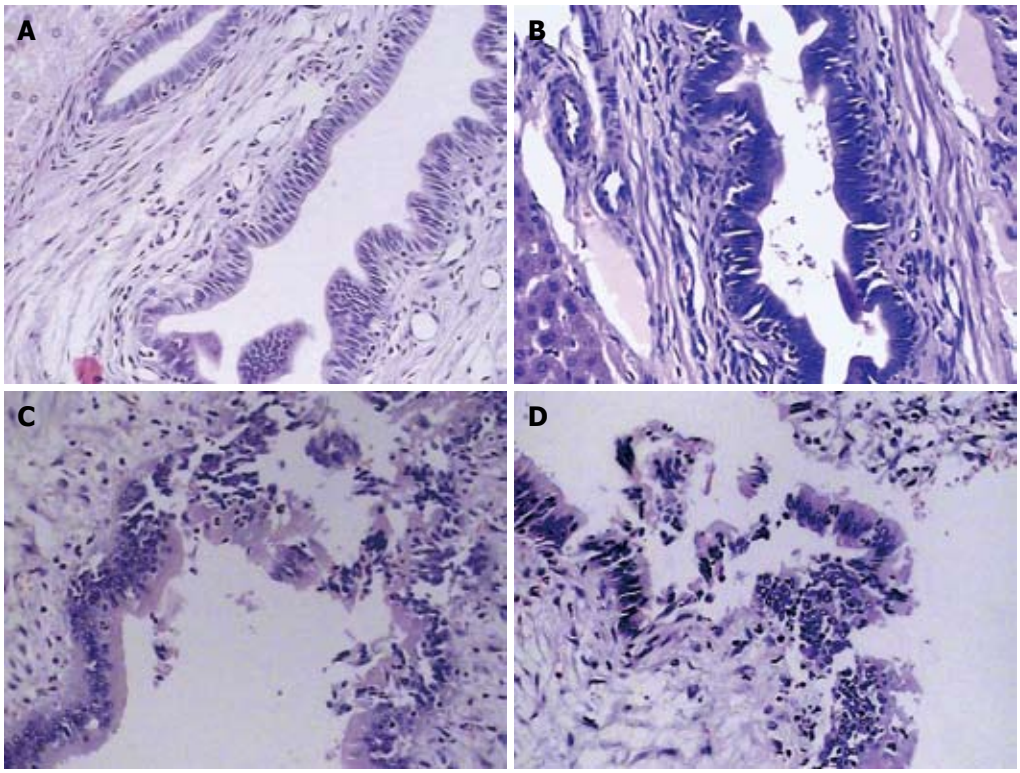

Figure 2 Histopathological manifestations of the four groups 4 wk after operation. A (SO): The intrahepatic bile duct epithelial cells were eumorphic and no cell necrosis was observed; B (ABO-1 h): The intrahepatic bile duct epithelial cells were almost normal. Small amounts of bile duct epithelial cells sloughed into the bile duct lumen; C (ABO-2 h): The intrahepatic bile duct epithelial cells were damaged obviously, and some of them were necrotic and sloughed into the bile duct lumen; D (ABO-3 h): The intrahepatic bile duct epithelial cells were damaged severely; many of them were necrotic and sloughed into the bile duct lumen. The normal epithelial structures disappeared (HE, × 200).

biliary lesion for studying the etiology, development and prophylaxis of ITBL.

Over the past years, several risk factors of this complication have been identified, strongly suggesting a multi-factorial origin, although the exact pathophysiological mechanism of ITBL is still unknown. The generally accepted risk factors include prolonged cold ischemic time warm ischemic time, reperfusion injury, disturbed blood flow in the peribiliary vascular plexus, ABO incompatibility, cytomegalovirus infection, chemokine polymorphism CCR5 delta 32, and bile salt-induced injury[2,5]. Obviously, ischemic injury is of vital importance for the occurrence of ITBL.

Several studies[6-8] have suggested that the peribiliary plexus (PBP) and hepatic artery branches are both the blood supply of the intrahepatic bile duct. Furthermore, it is demonstrated by scanning electron microscopy that there is no arterio-portal anastomosis in the liver of rabbits[9]. That is to say, the intrahepatic bile duct of rabbits do not receive their blood supply from portal vein. Therefore, in this study, by combined clamping of common bile duct and hepatic artery, as well as isolating the liver from all peripheral vascular connections, the blood supply of the intrahepatic bile duct was occluded nearly completely. After removing the clip, the intrahepatic bile duct underwent warm ischemia-reperfusion, which better simulated the clinical procedure of intrahepatic biliary warm ischemia and reperfusion injury in liver transplantation.

In this study, with the increase of the clamping time (1 h, 2 h, 3 h), it was found that intrahepatic biliary lesions were aggravated proportionally, as observed by biochemical indexes, cholangiography and histopathologicial examination. As for the clamping time, in group ABO-1 h, biliary lesions were mild and no imaging and histopathological changes were found, while in group ABO-3 h, the success rate of the animal model was only 60%, in spite of the obvious intrahepatic biliary lesion. However, significant biliary lesions and a high success rate of the model (100%) were both observed in group ABO-2 h. Therefore, combined clamping of the common bile duct and hepatic artery for 2 h was considered the optimal clamping time to establish the model of ischemic-type intrahepatic biliary lesion in rabbits.

Generally, the time from biliary ischemic necrosis and fibrosis to stricture is about 30 d after clinical liver transplantation. Zhao *et al*[10] established an animal model of biliary ischemic stenosis with clamping in mice and observed the significant extrahepatic biliary ischemic stenosis on day 21 after operation. Therefore, the time interval of 4 wk was chosen in this study for the initial observation time. It was not confirmed that the animals in group ABO-2 h underwent more significant intrahepatic stricture when prolonging the observation time after operation.

Overall, the advantages of this animal model included the following. (1) By combined clamping of the common bile duct and hepatic artery, as well as isolating the liver from all peripheral vascular connections, the intrahepatic bile duct is in complete warm ischemia, which can better reflect the clinical procedure of intrahepatic biliary warm ischemia and reperfusion injury in liver transplantation. (2) Surgery with occlusion of the common bile duct and hepatic artery and without that of portal vein, is easy to perform, and has less trauma and higher survival rate. (3) The common bile duct and hepatic artery of all the animals are clamped by the same microvascular clip, which can control

the strength and time precisely. (4) The animal model excluded the influence of other surgical operations (biliary anastomosis, hepatic artery anastomosis, T tube detaining), rejection, biliary cold conservation and drug toxicity. (5) The intrahepatic biliary anatomy, structure and microcirculation of the rabbits are similar to those of humans. Moreover, this animal is cheap and easy to obtain sufficient samples[11].

In conclusion, in the present study, by combined clamping of the common bile duct and hepatic artery for 2 h, producing the biliary ischemia-reperfusion injury, and raising the rabbits for 4 wk, the animal model of ischemic-type intrahepatic biliary lesion in rabbits was successfully established, which may provide a reliable technique for basic and clinical research into the etiology, development and prophylaxis of ischemic type intrahepatic biliary lesion after liver transplantation.

COMMENTS

Background

Biliary complications are a major cause of morbidity and graft failure in patients after orthotopic liver transplantation (OLT). The most troublesome is the so-called ischemic type biliary lesion (ITBL), which is one of the most important reasons for liver re-transplantation. Therefore, it is of clinical significance to establish an animal model of ischemic type intrahepatic biliary lesion for studying the etiology, development and prophylaxis of ITBL.

Research frontier

ITBL, with an incidence varying between 5% and 15% after OLT, is defined as non-anastomotic destruction of the graft's biliary tree after OLT. Although the exact pathophysiological mechanism of ITBL is still unknown, several risk factors of this often cumbersome complication have been identified, strongly suggesting a multi-factorial origin. Therefore, the etiology, development and prophylaxis of ITBL have been research hotspots.

Innovations and breakthrough

This animal model of ITBL is easy to establish, and has less trauma and a higher survival rate. Moreover, it can better reflect the clinical procedure of intrahepatic biliary warm ischemia and reperfusion injury in liver transplantation. In addition, the model excluded the influence of other operations, rejection and biliary cold conservation.

Applications

This animal model of ITBL may provide a reliable technique for basic and clinical research into the etiology, development and prophylaxis of ischemic type intrahepatic biliary lesion after liver transplantation.

Peer review

The manuscript is of interest as a measure of assessing ischemia of the liver and bile ducts, and the methods appear acceptable. It is an interesting study.

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Synergetic anticancer effect of combined gemcitabine and photodynamic therapy on pancreatic cancer *in vivo*

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Abstract

AIM: To investigate the anti-tumor effects of combined cytotoxic drug (gemcitabine) and photodynamic therapy (PDT) on human pancreatic cancer xenograft in nude mice.

METHODS: Human pancreatic cancer cell line SW1990 was used in the investigation of the *in vivo* effect of combined gemcitabine and PDT on human pancreatic cancer xenograft in mice. Sixty mice were randomly allocated into a control group (without treatment), photosensitizer treatment group (2 mg/kg photosan, without illumination), chemotherapy group (50 mg/kg gemcitabine i.p.), PDT group (2 mg/kg photosan + laser irradiation) and combined treatment group (photosan + chemotherapy), with 12 mice in each group. Tumor size was measured twice every week. Anti-tumor activity in different groups was evaluated by tumor growth inhibition (TGI).

RESULTS: No significant anti-tumor effect was observed either in photosensitizer treatment group or in chemotherapy group. PDT led to necrosis in cancer lesions and significantly reduced tumor volume compared with photosensitizer on day 6 and at the following time points after initialization of therapy (0.24 ± 0.15-0.49 ± 0.08 *vs* 0.43 ± 0.18-1.25 ± 0.09, $P < 0.05$). PDT significantly reduced tumor volume in combined treatment group compared with photosensitizer treatment group (0.12 ± 0.07-0.28 ± 0.12 *vs* 0.39 ± 0.15-1.20 ± 0.11, $P < 0.05$), small dose chemotherapy group (0.12 ± 0.07-0.28 ± 0.12 *vs* 0.32 ± 0.14-1.16 ± 0.08, $P < 0.05$) and control group (0.12 ± 0.07-0.28 ± 0.12 *vs* 0.43 ± 0.18-1.25 ± 0.09, $P < 0.05$). TGI was higher in the combined treatment group (82.42%) than in the PDT group (58.18%).

CONCLUSION: PDT has a significant anti-tumor effect, which is maintained for a short time and can be significantly enhanced by small doses of gemcitabine.

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Key words: Pancreatic carcinoma; Nude mice; Animal model; Photodynamic therapy; Gemcitabine

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INTRODUCTION

Pancreatic cancer remains a lethal disease. Most pancreatic cancer patients are at advanced stage when apparent symptoms occur. Pancreatic cancer patients undergoing resection at the time of initial diagnosis account for less than 20%[1], and the 5-year survival rates after complete and partial pancreatic resection are 15%-25%[2-3] and 8%-14%[4-5], respectively. Treatment modalities for inoperable patients are largely limited to radiotherapy, chemotherapy, or their combination. Pancreas is a retroperitoneal organ, with stomach, intestine, and spinal cord around it. Since the sensitivity of pancreatic cancer to radiotherapy is poor, and the tolerant dosage of pancreas tissue is low, radiotherapy does not lead to a convincing beneficial effect on survival. Chemotherapy is the main therapeutic method for advanced pancreatic cancer. 5-fluorouacil is probably

the most useful single agent for symptomatic relief[6]. Gemcitabine may also have a value for palliation[7-9]. Overall, the prognosis of pancreatic cancer patients is poor with a 1-year survival rate of about 10%[10]. Therefore, new treatment modalities are urgently needed.

Photodynamic therapy (PDT) produces localized tissue necrosis with light (most conveniently from a laser), after administration of a photosensitizing agent in the presence of oxygen[10-12], based on the use of photosensitizing compounds that localize quite selectively in neoplastic/hyperplastic tissues and become cytotoxic when exposed to light[12-14]. In view of the antitumor effect of single treatment, PDT is local. PDT in combination with surgery[15], radiotherapy[16] or chemotherapy[17], has become a subject of research. New photosensitizers with improved spectroscopic, photochemical and tissue-localizing properties and improved laser instrumentation have stimulated attempts to establish clinical protocols for incorporation of PDT into multi-treatment modalities[18-24].

Most studies on PDT to date have been on lesions of the skin or in the wall of hollow organs, but recent interest is more in its potential for treating lesions of solid organs such as the pancreas[10,11,20]. We have undertaken experiments on treating pancreatic cancer of mice with different doses of gemcitabine[25]. The results indicate that the growth of transplanted tumors can be inhibited by gemcitabine at 100 mg/kg, which shows severe side effects such as diarrhea, dehydration and loss of weight[25]. When gemcitabine at 100 mg/kg is used, the growth of transplanted tumors could not be controlled[25]. On the other hand, combined angiogenetic inhibitors can decrease side effects of gemcitabine, meanwhile the growth and metastasis of transplanted tumors are effectively inhibited[25].

In this study, gemcitabine was used as a cytotoxic drug. The cytotoxic and antitumor effects of combined gemcitabine and PDT were evaluated. Human pancreatic cancer cell line SW1990 was used in experiments to assess the effect of gemcitabine or PDT, or their combination, on pancreatic cancer in accordance with institutional guidelines.

MATERIALS AND METHODS

Tumor line, animals and drugs

SW1990, a high transferred human pancreatic cancer cell line (ATCC, Kyriazis MD, USA), was maintained in Laboratory of Sun Yat-Sen Memorial Hospital and serially passed in nude mice. Five- to six-week-old male BALB/c nude mice were obtained from Animal Center Sun Yat-Sen University. Photosan, a second generation of photosensitizer, was provided by Diolitec Pharmaceutical Company (Germany). Gemcitabine was provided by Lilly Pharmaceutical Company (USA).

Animal model and therapeutic group

Two male nude mice (6 wk of age) were inoculated subcutaneously with 0.5×10^7 SW1990 cells. Tumors in subcutaneous tissue were excised and tumor tissue was implanted subcutaneously in 60 nude mice.

A tumor model was established and observed for 10-14 d after implantation of tumor tissue. Tumor-bearing nude mice were divided into control group (group A), photosensitizer group (group B), chemotherapy group (group C), PDT group (group D), and combined group (group E), with 12 mice in each group.

Forty-eight hours after the mice had photosan, tumor masses in mice of the PDT group and combined group were exposed to light (λ = 630 nm, 120 J/cm^2) from a PDT630 semiconductor laser (Diolitec Pharmaceutical Company) for 20 min. Gemcitabine (50 mg/kg) was injected into the peritoneal cavity of mice in the combined group 1 h prior to light exposure and on days 3, 6 and 9 after light exposure. The same dose of gemcitabine was given to mice in the chemotherapy group at the same time point as in the combined group.

Data collection

All experimental mice were weighed and tumor diameters were measured with vernier calipers before treatment and twice a week after treatment. On day 21 after treatment, all experimental mice were sacrificed with their tumors removed and weighed to obtain tumor weight (TW). Tumor volume (TV) was determined according to the formula: $TV = 3/4 \times \pi \times (b/2)^2 \times a/2$, where a is the length and b is the height of the tumor. Antitumor activity was evaluated by tumor growth inhibition (TGI), calculated according to the formula: $TGI = (1 - TW_T/TW_C) \times 100\%$ in treated (T) and control (C) mice.

Statistical analysis

Data analysis was performed using SPSS11.0 statistical package (SPSS, Chicago, USA). Tumor response to treatment was compared using two-way ANOVA and Student-Neuman-Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

Tumor volume

Tumor volume increased with time after treatment in the photosensitizer group, small dose chemotherapy group and control group (Table 1 and Figure 1). Tumor volume had no significant difference at the same time point in three groups (ANOVA).

PDT led to necrosis in cancer lesions. Partial tumor necrotic tissue was exfoliated and a necrotic edge of volcano-like uplift was formed 1 wk after treatment. Tumor volume was significantly reduced in PDT treatment group compared with the photosensitizer treatment group and control group at different time points after initialization of therapy (ANOVA, $P < 0.05$). No significant difference in tumor volume was found on days 3, 6, 9 and 12, but tumor volume increased significantly on days 15, 18 and 21 ($P < 0.05$) in the PDT group after treatment.

Table1 Tumor volume in different groups after treatment with PDT and/or gemcitabine (cm^3) (mean ± SE)

Groups	Pre-therapy	3 d	6 d	9 d	12 d	15 d	18 d	21 d	*P*
A	0.14 ± 0.09	0.26 ± 0.13	0.43 ± 0.18	0.56 ± 0.23	0.66 ± 0.23	0.80 ± 0.10	1.01 ± 0.12	1.25 ± 0.09	< 0.01
B	0.12 ± 0.06	0.26 ± 0.11	0.39 ± 0.15	0.51 ± 0.18	0.62 ± 0.17	0.75 ± 0.09	0.93 ± 0.08	1.20 ± 0.11	< 0.01
C	0.13 ± 0.07	0.23 ± 0.10	0.32 ± 0.14	0.44 ± 0.14	0.57 ± 0.12	0.72 ± 0.10	0.91 ± 0.12	1.16 ± 0.08	< 0.01
D	0.14 ± 0.08	0.22 ± 0.12	0.24 ± 0.15	0.24 ± 0.16	0.28 ± 0.12	0.35 ± 0.10	0.42 ± 0.12	0.49 ± 0.08	< 0.01
E	0.12 ± 0.07	0.13 ± 0.09	0.14 ± 0.10	0.15 ± 0.09	0.17 ± 0.08	0.18 ± 0.10	0.22 ± 0.10	0.28 ± 0.12	< 0.01
P	0.951	0.038	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	

Variance-test, $P > 0.05$.

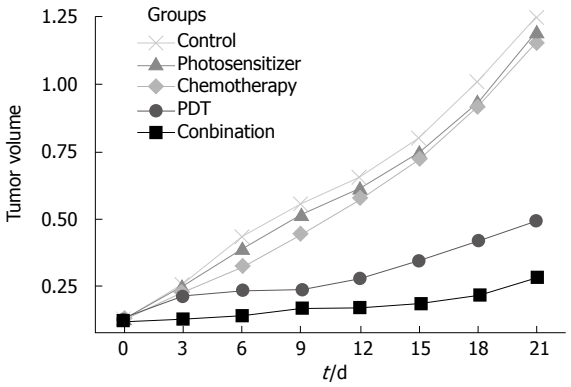

Figure 1 Tumor volume in different groups after treatment with PDT and/or gemcitabine (cm^3) (mean ± SE).

Tumor volume was significantly decreased in the combined PDT and gemcitabine treatment group compared with photosensitizer treatment group, small dose chemotherapy group and control group ($P < 0.05$). Tumor volume was significantly smaller in the combined treatment group than in the PDT group at different time points after treatment.

Tumor weight and TGI

Tumor weight and TGI of mice in five groups are listed in Table 2. Tumor weights of mice in the combination group (0.29 ± 0.20 g) and PDT group (0.69 ± 0.23 g) were significantly lower than those in the photosensitizer treatment group (1.62 ± 0.12 g), chemotherapy group (1.37 ± 0.19 g) and control group (1.65 ± 0.21 g) ($P < 0.01$). Tumor weights of mice in the combined group were obviously lower than those in the PDT group ($P < 0.01$). The mean tumor weight of mice in the two groups was significantly different ($P < 0.01$). Tumor weights were not significantly different in the photosensitizer treatment group, small dose chemotherapy group and control group. TGI (82.42%) was higher in the combined treatment group than in the PDT treatment group (58.18%).

Toxicity

The mice in four experimental groups had a loss of weight during the experiments. The weight in the four experimental groups was not significantly different from that in the control group.

Four treatment modalities did not induce signs of toxicity such as diarrhea and vomiting. No treatment-related deaths occurred. Mice in the PDT group and combined treatment group had no skin photosensitivity to light.

Table 2 Tumor weight (g) and TGI (%) response to PDT and/or gemcitabine

Group	Animals (*n*)	TW (mean ± SE)	TGI
A	12	1.65 ± 0.21	-
B	12	1.62 ± 0.12	1.80
C	12	1.37 ± 0.19	17.00
D	12	0.69 ± 0.23	58.18
E	12	0.29 ± 0.20	82.42
P	-	< 0.01	-

Variance-test, $P = 0.1$.

DISCUSSION

PDT may be defined as a treatment based on an oxygen-dependent reaction between a photosensitizing dye and light, that is, the light combination of two absolutely non-toxic elements, drug and light, in the presence of oxygen, can result in selective destruction of tissue[11,26]. The technique consists of administration of a tumor-localizing photosensitizing agent, which most often requires metabolic synthesis followed by activation of the agent by light with a specified wavelength. Photosensitizing agents used in PDT are macromolecular materials, which contribute to preferential location in neoplastic tissues and delay clearance of neoplastic tissues[27]. Therefore, PDT aims at a sequence of photochemical and photobiological processes that cause irreversible damage to tumor tissues and little damage to connective tissues, and maintain the mechanical integrity of organs[12,24]. It was reported that PDT has a selective effect on malignant pancreas but no significant effect on normal pancreas, and could well match other treatment modalities, except for radical surgery[28]. In a pilot clinical trail, Bown *et al*[10] used PDT in the palliative treatment of 16 patients with cancers in the pancreatic head that could not be treated with surgery because of the advanced nature of the disease or the general condition of the patients, and they concluded that PDT may be of value for treating localized cancers in patients who are poor candidates for definitive surgery or in whom the location of tumor makes pancreatic resection inappropriate. Abulafi *et al*[29] and Tseng *et al*[30] reported that patients with pancreatic and ampullary carcinoma

who are not suitable for surgery should be treated with PDT, since PDT is both feasible and safe for small tumors.

PDT, on the other hand, has some disadvantages and limitations. Little information is currently available concerning the uptake of photosensitizer by pancreas or pancreatic cancer. Local spread of photodynamic agents to vital organs is common, and may cause perforation of the duodenum and jejunum, leading to death after treatment with PDT[11,31]. In addition, large tumor mass limits PDT to penetrate into the effective depth of tissue and needs multiple interstitial optical fibers to increase its volume[32]. Therefore, the ability of chemotherapeutic agents to enhance the effects of PDT in cell culture and transplantable mouse tumors has been studied by several groups[20-22]. Kirveliene *et al*[20] used murine hepatoma MH-22A to investigate *in vitro* and *in vivo* cytotoxic and anti-tumor effects of doxorubicin (Dox), a conventional anticancer drug, and PDT, showing that Dox potentiates therapeutic efficacy of PDT and *vice versa*, and that the degree of potentiation is influenced by Dox. Peterson *et al*[21] and Snyder *et al*[22] reported that combined treatment with PDT and Dox is more effective than treatment with either PDT or Dox alone. *In vitro* studies have revealed a significant effect of fluoropyrimidines[18] and mitomycin C[19] on the viability of mTHPC-photosensitized cells. Some studies focused on the effects of PDT on pancreas cancer[10,11]. As we know, no study about the effect of therapy with photodynamic-cytotoxic agents on pancreatic cancer has been reported.

Gemcitabine is an active nucleoside analogue against a wide variety of cancers, including non-small cell lung cancer and pancreatic cancer[7-9]. Gemcitabine, acting as an antimetabolite, can inhibit ribonucleotide reductase and DNA synthesis, and induce apoptosis[33]. Gemcitabine today remains a first-line drug for patients with advanced pancreatic cancer[7-9]. However, it has a narrow therapeutic index due to rapid enzyme deamination by deoxytidine deaminase into its corresponding inactive uracil derivative, and can also induce drug resistance[34,35]. Therefore, a high dose of gemcitabine is needed to achieve the desired therapeutic response with different adverse effects[36]. To overcome such drawbacks, based on the relation between quantity and effect of gemcitabine[25], we used photosan and gemcitabine as representatives of photosensitizing and cytotoxic agents to investigate the cytotoxic and antitumor effects of gemcitabine and PDT on pancreatic cancer *in vivo*. The results indicate that small dose gemcitabine or photosensitizer can not inhibit the growth of pancreatic cancer. PDT had a significant anti-tumor effect, but lasted a short time. Photodynamic-cytotoxic therapy (small dose gemcitabine) could significantly inhibit the growth of pancreatic cancer, and showed a relative long-duration anti-tumor effect compared with PDT. The inhibition rate of photodynamic-cytotoxic agents and PDT for tumors was 82.42% and 58.18%, respectively. The four treatment modalities did not induce any signs of toxicity such as diarrhea and vomiting. No treatment-related death occurred. Animals in the PDT group and combined treatment group had no skin photosensitivity to light.

In conclusion, low dose gemcitabine increases the anticancer effect of PDT with no obvious adverse effects. Combined PDT and gemcitabine can be used in treatment of pancreatic cancer in patients who are poor candidates for surgery.

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COMMENTS

Background

Most patients with pancreatic cancer are at advanced stage when apparent symptoms occur. Treatment of pancreatic cancer remains a great challenge, and new treatment modalities are urgently needed. In this study, gemcitabine was used as a cytotoxic drug, and cytotoxic and antitumor effects of combined gemcitabine and photodynamic therapy (PDT) were evaluated.

Research frontiers

Gemcitabine may have a value in treatment of pancreatic cancer. Recently, more studies on PDT have been focused on solid pancreatic caner. The prognosis of pancreatic cancer is poor. This is the first report on the synergetic anticancer effect of combined gemcitabine and PDT on pancreatic cancer *in vivo*.

Innovations and breakthroughs

The results of this study indicate that PDT-cytotoxic therapy (small dose gemcitabine) could significantly inhibit the growth of pancreatic cancer, and showed a relative long duration of anti-tumor effect compared with PDT. This study first demonstrated that low dose gemcitabine could increase the anticancer effect of PDT with no obvious adverse effects.

Applications

Combined PDT and gemcitabine therapy can be used in treatment of patients who are poor candidates for surgery.

Terminology

PDT is a way to produce local tissue necrosis with light (most conveniently from a laser) after administration of a photosensitizing agent in the presence of oxygen. PDT is based on the use of photosensitizing compounds that localize quite selectively in neoplastic/hyperplastic tissues and become cytotoxic when exposed to light.

Peer review

The study revealed that PDT can significantly inhibit the growth of pancreatic cancer, and its effect could be significantly enhanced by a small dose of gemcitabine. The findings are of great interest and provide a foundation for its application in clinical practice. The data are reliable and valuable.

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BRIEF ARTICLES

Diagnosis of chest pain with foregut symptoms in Chinese patients

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Abstract

AIM: To evaluate the diagnosis of chest pain with foregut symptoms in Chinese patients.

METHODS: Esophageal manometric studies, 24-h introesophageal pH monitoring and 24-h electrocardiograms (Holter electrocardiography) were performed in 61 patients with chest pain.

RESULTS: Thirty-nine patients were diagnosed with non-specific esophageal motility disorders (29 patients with abnormal gastroesophageal reflux and eight patients with myocardial ischemia). Five patients had diffuse spasm of the esophagus plus abnormal gastroesophageal reflux (two patients had concomitant myocardial ischemia), and one patient was diagnosed with nutcracker esophagus.

CONCLUSION: The esophageal manometric studies, 24-h intra-esophageal pH monitoring and Holter electrocardiography are significant for the differential diagnosis of chest pain, particularly in patients with foregut symptoms. In cases of esophageal motility disorders, pathological gastroesophageal reflux may be a major cause of chest pain with non-specific esophageal motility disorders. Spasm of the esophageal smooth muscle might affect the heart-coronary smooth muscle, leading to myocardial ischemia.

INTRODUCTION

Recent reports[1-2] have indicated that recurrent chest pain is often a result of esophageal motility disorders or gastroesophageal reflux diseases (GERD), which is known as esophageal chest pain. Esophageal chest pain is very similar to symptoms seen during myocardial ischemia. It is also observed in patients with coronary artery disease with similar incidence (i.e. linked angina)[3]. As a result, differential diagnosis is often difficult and many patients with esophageal chest pain are misdiagnosed as having coronary artery disease[4]. Hence, the percentage of patients that are correctly treated and cured is relatively low. It is well known that esophageal manometry and 24-h intra-esophageal pH monitoring provide accurate diagnoses of esophageal motility disorders. As an incentive, Holter electrocardiography is more economical for these patients from developing countries compared to coronary artery opacification.

Previous reports have focused on the diagnosis of unclear chest pain via both coronary artery 24-h intra-esophageal pH monitoring and coronary artery opacification, which is very expensive to the patient, particularly in developing countries. In addition, very few studies have shown the efficacy of combined esophageal manometry, 24-h intra-esophageal pH monitoring, and electrocardiograms from Holter

monitoring and the results have been inconclusive. Paterson *et al*[5] reported that there was no correlation between the incidence of chest pain and changes in esophageal manometry, 24-h intra-esophageal pH monitoring, and Holter electrocardiography. Wright *et al*[6] reported that physiologic gastroesophageal reflux does not induce electrocardiographic changes from Holter monitoring. However, Dobrzycki *et al*[7] and Patai *et al*[8] reported distinguishable alterations in 24-h intra-esophageal pH monitoring and Holter electrocardiography during chest pain. As a result, it was thought that simultaneous 24-h esophageal pH manometry and electrocardiograms from Holter monitoring could contribute to the diagnosis of atypical chest pain. However, up to now, many questions remain unaddressed. Can esophageal motility disorders affect myocardial ischemia? Can GERD affect myocardial ischemia? All of these diseases can cause chest pain, so it is unclear how to differentiate between cause and effect in these patients. Is there any clinical significance to the combination of these three types of monitoring in the differential diagnosis of unclear chest pain? Here, we present our experience in the combined application of monitoring over the past 6 years.

MATERIALS AND METHODS

Patients

From September 2001 to May 2007, 61 Chinese patients with chest pain from the thoracic surgery department were enrolled into this study. The patients were both male (27) and female (34), ranging in age from 18 to 69 years (average age: 45.5 years). All patients had complaints of varying degrees of chest pain and most had symptoms of retrosternal pain or backache, ranging in duration from 60 d to 5 years (average duration of pain: 14.5 mo). Twenty patients had intermittent dysphagia and 40 had other symptoms such as regurgitation. There were no abnormalities in their hemogram, chest X-rays, routine electrocardiograms or esophago-gastroscopy.

Methods

Esophageal motility was studied by standard water perfusion and stationary manometry (Medtronic DPT-6000, Smith Medical, Sweden) with computer-assisted analysis (Polygram 2.0, Smith Medical, Sweden) of the tracings according to a previously published protocol[9-10]. Briefly, a station pull-through technique was applied, and measurements were made at the pressure levels of the lower esophageal sphincter (LESP), the relaxation rate of the lower esophageal sphincter (LESRR), the esophageal body, the upper esophageal sphincter and the pharynx.

Twenty-four-hour intra-esophageal pH was monitored using the classical DeMeester criteria[11]. A single channel, nasoesophageal, antimony pH-probe (Synectics Medical, Sweden) was positioned 5 cm above the lower esophageal sphincter and was connected to a portable data acquisition system (Digitrapper Mark II Gold, Synectics Medical, Sweden). Following 24-h intra-esophageal pH monitoring, chest leads at CM1, CM5 and CMF of a digital ambulatory 24-h Holter monitor (Life Card CF, Reynolds Medical, England) were positioned. Holter studies and 24-h pH monitoring were performed simultaneously. Following 24-h monitoring, data from the two monitors were analyzed by software (supplied by Life Card CF, Reynolds Medical, England and Digitrapper Mark Ⅱ Gold, Synectics Medical, Sweden).

RESULTS

Results interpretation

Esophageal contraction waves following swallowing were classified as (1) peristaltic, (2) simultaneous, (3) interrupted, or (4) dropped. Primary esophageal motility disorders were classified according to Chinese standards[12]: (1) Diffuse esophageal spasm; (2) Nutcracker esophagus; or (3) Non-specific esophageal motility disorders. Abnormal gastro-esophageal reflux was considered when the score was > 14.72. If the decreasing amplitude of the ST segment was above 0.1 mV, chest pain arousing from myocardial ischemia was considered, according to an electrocardiogram obtained from the Holter monitor.

Results of combined examination

In the present case report, 45 of 61 patients had different types of esophageal motility disorders (Table 1). The episodes of pain are shown in Table 1. Results of esophageal manometric studies and 24-h intra-esophageal pH monitoring are presented in Table 2.

Eight patients were diagnosed with myocardial ischemia and non-specific esophageal motility disorders. Two patients were diagnosed with myocardial ischemia and diffuse spasm of the esophagus. Of the above 10 patients, eight had myocardial ischemia, which occurred with simultaneous abnormal gastroesophageal reflux (GERD) (Figure 1).

DISCUSSION

Recurrent chest pain is typically general and difficult to identify. Shrestha *et al*[4] reported that 30% of non-cardiac chest pain was caused by esophageal diseases such as GERD. Since the innervation and location of the esophageal nervous system in the body overlaps with the cardiac nervous system, symptoms are often similar. As a result, patients with esophageal chest pain are often misdiagnosed as having coronary artery disease[13]. Therefore, it is important for physicians to pay particular attention to the differential diagnosis of inconclusive esophageal chest pain.

As for the differential diagnosis of chest pain, there are very few studies investigating combined 24-h intra-esophageal pH and electrocardiograms from Holter monitoring. Several studies[5-6] have found that there is minimal correlation between the incidence of

Table 1 Results of esophageal manometric studies, 24-h intra-esophageal pH monitoring and electrocardiograms from Holter monitoring in 77 patients

Diagnosis	Number cases and pain episodes		Cases combined with abnormal gastroesophageal reflux			Cases combined with myocardial ischemia		
	Cases	Pain episodes	Cases	Pain episodes	Pain episodes with changes in 24-h pH monitoring	Cases	Pain episodes	Pain episodes with changes in Holter monitoring
Non-specific esophageal motility disorders	39	312	29	235	156	8	61	10
Diffuse spasm of esophagus	5	65	5	65	53	2	25	13
Nutcracker esophagus	1	18	0	18	0	0	0	0
Normal case	16	83	0	0	0	0	0	0
Consolidation of table	61	478	34	366	0	10	86	23

Table 2 Results of esophageal manometric studies and 24-h intra-esophageal pH monitoring

	Cases	LESP	LESRR	Overall length of LES	Abdominal length of LES	Swallow waves	pH monitoring DeMeester scores
Non-specific esophageal motility disorders	39	Reduced in 29 patients (9.32 ± 1.53 mmHg)	Reduced in 28 patients (52.18 ± 20.51%)	Reduced in 21 patients (2.3 ± 0.1 cm)	Reduced in 18 patients (1.2 ± 0.1 cm)	39 patients with simultaneous non-transmitted waves	60.2 ± 12.4
Diffuse spasm of esophagus	5	Normal in 5 patients (18.35 ± 2.92 mmHg)	Reduced in 5 patients (30.50% ± 6.65%)	Normal in 5 patients (3.3 ± 0.3 cm)	Normal in 5 patients (2.0 ± 0.1 cm)	20% or more simultaneous contractions in response to wet swallows appearing in all five patients	80.4 ± 35.5
Nutcracker esophagus	1	12.2 mmHg	64.0%	4.0 cm	2.5 cm	High amplitude contracting wave appearing above 229.7 mmHg	

chest pain and changes in esophageal dysfunction and myocardial ischemia monitoring, while others have reported the opposite[7,8]. Therefore, in the present study, we reassessed the significance of the combined monitoring. In our study, 45 of 61 patients had esophageal disorders (the rate was 73.7%, which was rather high since the patients were recruited from the thoracic surgery outpatient clinic and some of them had pre-existing upper gastrointestinal symptoms such as dysphagia or regurgitation), and 10 (16.4%) patients had myocardial ischemia. We think that the main reason for the weak correlation between incidences of chest pain and changes in the combined monitoring reported by Paterson *et al*[5] and Wright *et al*[6] may be as follows: (1) the number of samples that were selected randomly was relatively small; and (2) there may have been an unknown heterogeneity of patient populations.

Esophageal motility disorders are the most common cause of esophageal chest pain[14]. Non-transmitted contraction waves appeared in all of the 39 patients with non-specific esophageal motility disorders, while 29 patients had abnormal gastroesophageal reflux. Among the total 235 pain episodes, 156 had changes in 24-h pH monitoring, indicating that abnormal gastroesophageal reflux may be the main cause of chest pain in these patients, confirmed by the observation by Patai *et al*[8] showing that proton pump inhibition with omeprazole alleviated gastroesophageal reflux as well as spontaneous chest pain. Interestingly, exercise potentiated the effect of intra-esophageal pH monitoring on electrocardiogram abnormalities (Badzynski *et al*[15]).

In the five patients with diffuse spasm of the esophagus, 20% or more had simultaneous contractions in response to wet swallows. However, some degree of peristaltic function was retained and the differential diagnosis could be made between diffuse spasm of the esophagus and achalasia. All of the five cases were defined as secondary diffuse spasm of the esophagus due to abnormal gastroesophageal reflux[13]. Two patients were diagnosed as having simultaneous diffuse spasm of the esophagus, myocardial ischemia, and GERD. We hereby hypothesize that diffuse spasm of the esophageal smooth muscle might affect spasm of the heart-coronary smooth muscle, leading to myocardial ischemia. All of these could be caused by a disturbance of the nervous system controlling the esophagus and cardiovascular system. It is interesting that this presumption is supported by Manfrini *et al*[16], in which esophageal spasm was considered to be related to myocardial ischemia ($P < 0.05$). Bidirectional analysis of causal effects showed that the influence between esophageal and coronary spasms was mutual and reciprocal in seven patients with variant angina. In two patients in our study, 25 pain episodes occurred with eight episodes causing changes in 24-h pH and Holter monitoring. This fact indicates that myocardial ischemia may occur with GERD, especially in patients with diffuse spasm of the esophagus.

Amplitude of the contractive wave in the patients with nutcracker esophagus was 229.7 mmHg, consistent with the diagnostic criteria[17].

Hence, combined monitoring of esophageal manometry, 24-h intra-esophageal pH and electrocardiograms from Holter monitoring are very

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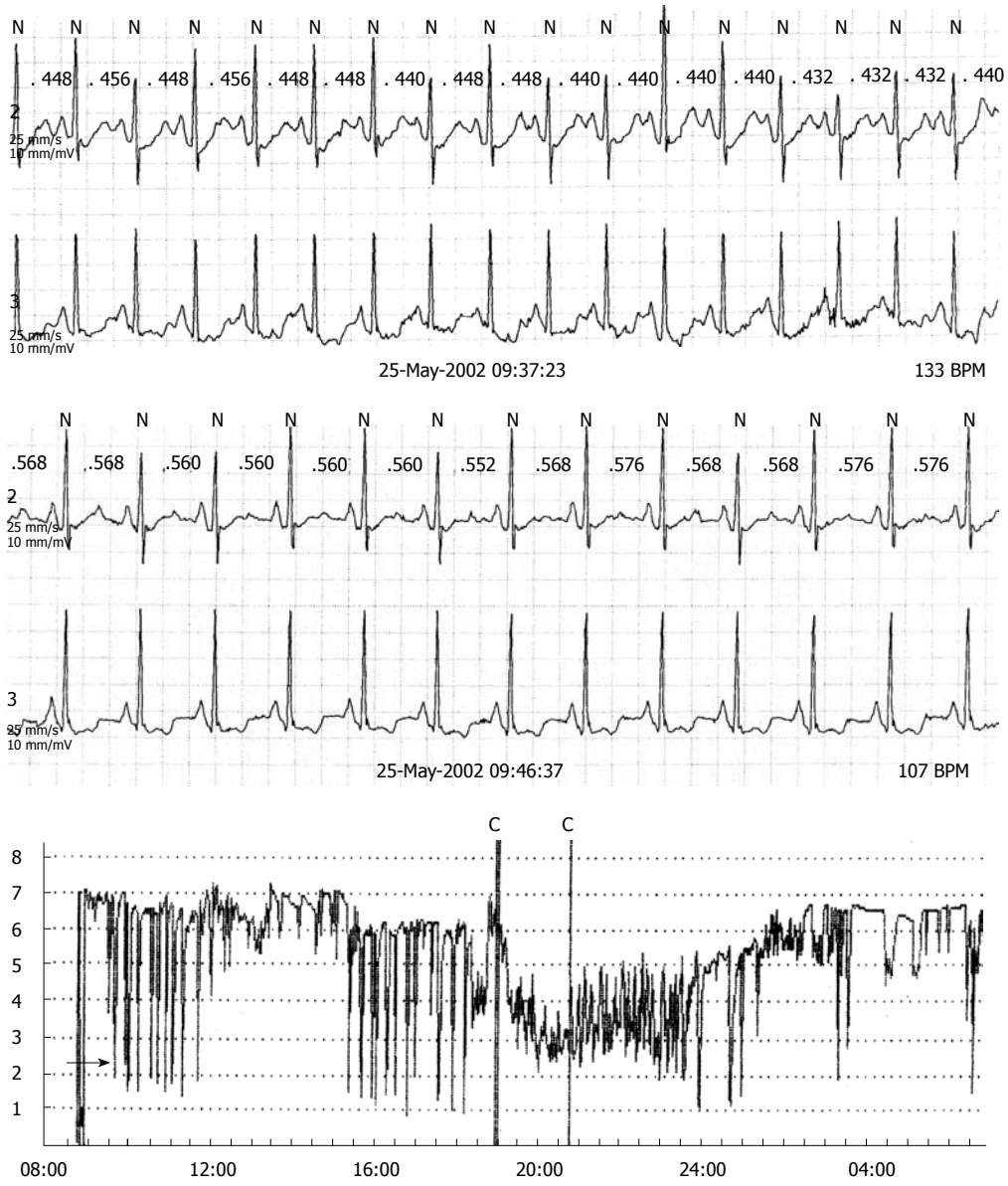
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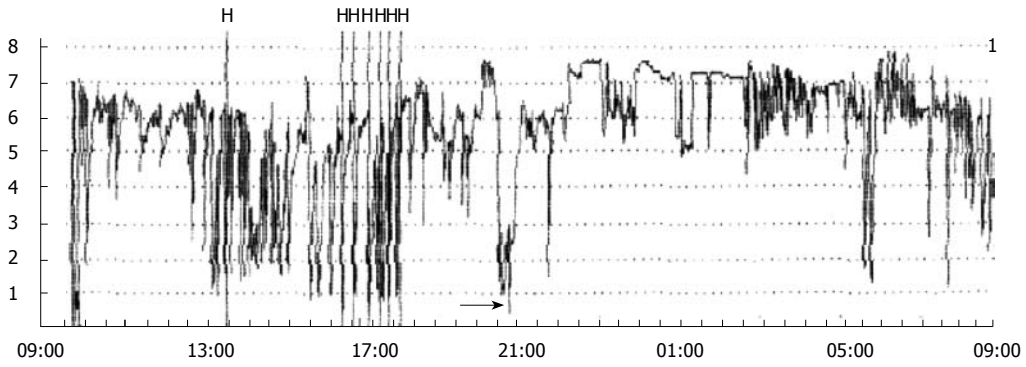

Figure 1 Curves of 24-h intra-esophageal pH monitoring and Holter electrocardiography in a patient with abnormal gastroesophageal reflux show the decreasing amplitude of the ST segment. A: Ranged from 0.05 mV to 0.15 mV in 9 min. The results also indicate that abnormal gastroesophageal reflux occurred during this period; B: 0.1 mV to 0.3 mV in from 12:38:07 to 20:50:02. The results also indicate that abnormal gastro-esophageal reflux occurred during this period.

significant for the diagnosis of recurrent chest pain, particularly for patients with foregut symptoms. We therefore, suggest that the diagnostic procedures of cloudy chest pain should be undertaken as follows. Firstly, the patients with cloudy chest pain should undergo routine examination including hemogram, chest X-rays, routine electrocardiogram and esophagogastroscopy, in order to exclude severe diseases such as tumor or myocardial infarction. Secondly, the combined examinations should be recommended to the patients without the positive results of the routine examinations and with foregut symptoms as the "final step" in the diagnostic procedures. The detection rate is satisfactorily high, as shown in our cases. In conclusion, as an added incentive, the combined monitoring is very cost-effective for the patients, with a total cost of approximately 50 US dollars.

COMMENTS

Background

Studies on unclear chest pain are timely and important with the growing age of the world's population. However, very few studies have been performed about esophageal manometric studies, 24-h intra-esophageal pH monitoring and a Holter electrocardiography for the differential diagnosis of chest pain caused by esophageal dysfunctional and/or myocardial ischemia. Interestingly, the results of published papers on the combined monitoring have been inconclusive.

Research frontiers

The aim of the present study was to evaluate the significance of combined monitoring in the diagnosis of chest pain with foregut symptoms in Chinese patients.

Innovations and breakthroughs

The study indicated that spasm of the esophageal smooth muscle might affect the heart-coronary smooth muscle, leading to myocardial ischemia. The combination of esophageal manometric studies, 24-h intra-esophageal pH monitoring and Holter electrocardiography are significant for the differential diagnosis of chest pain, particularly with foregut symptoms. As an added incentive, combined monitoring is very cost-effective for the patients, especially those from developing countries.

Peer review

The manuscript describes an interesting study that aimed to demonstrate the etiology of chest pain. In fact, it is well known that non-cardiac chest pain is associated with GERD in about 50% of cases and that spastic esophageal motility disorders are related to chest pain. This study revisits the conflicting situation concerning the precise primary cause of chest pain, especially in patients in whom myocardial ischemia, GERD and esophageal spastic motility disorders are simultaneously found. As the authors pointed out, it has been suggested that spastic motility disorders may cause myocardial ischemia.

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BRIEF ARTICLES

Combined therapy with transcatheter arterial chemoembolization and percutaneous microwave coagulation for small hepatocellular carcinoma

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Abstract

AIM: To assess the efficacy of combined transcatheter arterial chemoembolization (TACE) and percutaneous microwave coagulation therapy (PMCT) for small hepatocellular carcinoma (HCC).

METHODS: Thirty-five patients with a total of 41 HCC nodules (≤ 3 cm in diameter) were treated with TACE followed by computed tomograghy (CT)-guided percutaneous microwave coagulation therapy (PMCT) within 1-3 wk.

RESULTS: By biopsies and enhanced CT scans, complete necrosis of the tumor and 3-5 mm of the surrounding non-cancerous area were observed in 34 foci. In seven foci, incomplete necrosis of the surrounding parenchyma was observed. Serum alpha-fetoprotein (AFP) levels returned to normal 10 d after treatment in 25 patients who originally had high serum AFP levels. The follow-up period was 6-31 mo, and all patients remained alive. One patient had a recurrence in the subsegments of the liver, and another patient had a recurrence near the original lesion.

CONCLUSION: Combined therapy with TACE and PMCT is a safe and effective treatment without severe complications for small HCC.

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Key words: Liver neoplasms; Therapy; Hepatocellular carcinoma; Transcatheter arterial chemoembolization; Microwave coagulation therapy; Percutaneous local treatment

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

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INTRODUCTION

Surgical resection[1], transcatheter arterial chemoembolization (TACE), and percutaneous ethanol injection (PEI)[2-4] are effective therapies for small hepatocellular carcinoma (HCC). However, for patients with poor liver functions, hepatectomy is not the first treatment choice. TACE is not effective for HCC with a poor blood supply[5], and PEI only yields incomplete necrosis of the tumor due to the uneven distribution of ethanol[6]. Although percutaneous microwave coagulation therapy (PMCT) can lead to complete necrosis of the hepatocarcinoma cells, the induced area of necrosis is small[7,8]. TACE has the advantage of reducing the local blood supply of the tumor foci, resulting in tissue necrosis and inflammatory edema. Therefore, it can decrease the cooling effect of blood flow on the heating action of microwaves, and enhance the coagulation action of microwaves[9]. In the present study, we investigate the therapeutic efficacy of the combied therapy of TACE and PMCT for the treatment of small HCC.

MATERIALS AND METHODS

Patient data

A total of 35 patients received the combined therapy of TACE and PMCT in our hospital between April 2005 and May 2007. These patients comprised 27 males

and eight females, with an age range of 27-78 years, and a mean age of 56.69 ± 14.02 years. All patients had a history of hepatitis B and liver cirrhosis, with liver functions categorized as class A by Child-Pugh classification. All of the cases met the diagnostic criteria of primary HCC. Twenty-eight cases were confirmed by pathological examination of puncture-biopsied specimens under the guidance of computed tomograghy (CT) before PMCT. Twenty-five cases had elevated levels of serum alpha-fetoprotein (AFP). The size of the tumor focus was determined by measurement of the largest and shortest diameters of the largest section. All tumor foci were less than 3 cm in diameter.

Therapeutic methods

All patients were initially treated with TACE. Hepatic artery angiography was performed using the Seldinger technique. Femoral arterial catheterization was conducted through the common hepatic artery or proper hepatic artery, and the location, number, size, and blood supply of the tumors were evaluated. Subsequently, a microcatheter was super-selectively inserted into the hepatic lobe or hepatic segmental artery branch, and mixed suspensions of iodized oil (3-6 mL) and epirubicin (20-30 mg) were infused into the artery through the catheter. Finally, gelatin sponge particles were infused to embolize the artery until the arterial blood flow supplying the tumor was completely blocked.

PMCT was initiated 1-3 wk after TACE. The microwave therapeutic device, FORSEA MTC-3 for inter-tissue tumor treatment, was produced by Nanjing Qinghai Microwave Electric Institute (Nanjing, China). The diameter of its electrode needle was 14 G, and the instrument was cooled by a cold water cycling system. To relieve pain in the patients, dolantin (100 mg) and valium (10 mg) were injected intramuscularly 5 min before the PMCT.

The puncture site and pathway were determined under the guidance of CT. After anesthetizing the puncture site with 2% lidocaine, a 12 G guide-needle was placed at its opposite side, across the tumor focus. The electrode needle of the microwave device was connected to the output machine of the microwave instrument via a flexible coaxial cable, and the cooling water tube was connected. The constant-flow pump was switched on to test the functioning of the cold water cycling system. The inner needle of the guide-needle was withdrawn, and the electrode needle of the microwave machine was inserted through the outer needle of the guide to place the electrode in the tumor area. The microwave power was set at 60-70 W. The coagulation time for each focus was 10-15 min, and the coagulation area covering the tumor focus and its surrounding area measured 5 mm or more. If a single needle coagulation treatment did not produce satisfactory results, a second PMCT therapy was conducted a week later.

After TACE and PMCT treatment, liver protection, anti-inflammatory and sedation therapies were prescribed. A follow-up study by repeat CT (plain and enhanced) and serum AFP level measurement was conducted once every 1-2 mo.

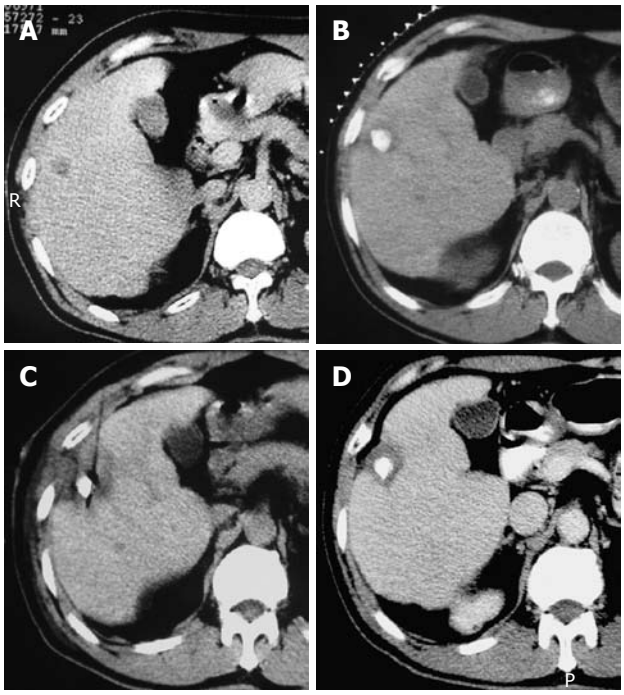

Figure 1 Pathological examinations of biopsied specimens and enhanced CT after PMCT showed complete tumor necrosis in 34 foci, together with complete ring-shaped necrosis of the surrounding non-cancerous hepatic parenchymal tissue, measuring 3-5 mm in width. A: CT scan of a primary HCC in the right anterior lobe of the liver, with a diameter of 1.6 cm × 1.4 cm; B: After TACE, the accumulation of iodized oil in the tumor area was satisfactory; C: PMCT was initiated 17 d later; D: One month after PMCT, an enhanced CT scan showed a complete non-enhanced area of the tumor, and a non-cancerous ring-shaped area surrounding the tumor (measuring 4-5 mm), indicating complete necrosis of the tumor lesion.

RESULTS

One to three weeks after all 41 foci were treated with TACE, these foci received PMCT treatment once. Among them, six foci were treated with a second PMCT within 1 wk.

Pathological examinations of biopsied specimens and enhanced CT after PMCT showed complete tumor necrosis in 34 foci, together with complete ring-shaped necrosis of the surrounding non-cancerous hepatic parenchymal tissue, measuring 3-5 mm in width (Figure 1). Seven other foci showed complete necrosis of the tumor, with incomplete necrosis of the surrounding non-cancerous hepatic parenchyma.

Among the 35 patients, serum AFP levels were elevated in 25; the AFP levels in these 25 patients returned to normal within 10 d after treatment.

Post-embolization symptoms included fever, pain, nausea, and vomiting after TACE, all of which were relieved by anti-inflammatory, liver protection, and sedation treatment. In the process of PMCT, most patients complained of local burning pain, which was generally tolerable, but in some patients, the microwave coagulation therapy had to be carried out intermittently.

After PMCT, some patients reported having fever and local pain, but these were not severe. Complications such as hemorrhage, subcapsular hematoma of the liver, or biliary duct damage were not found, nor was local dissemination of cancer cells along the puncture needle pathway.

All patients were followed up for 6-31 mo. No recurrence was found in 33 patients. One patient had a gradual increase of serum AFP levels 10 mo after the treatment, but repeat CT and magnetic resonance imaging (MRI) examinations revealed no recurrence. However, hepatic arteriography detected new tumor foci in the segments of the liver. Another patient showed an elevation of serum AFP levels 8 mo after treatment, and a CT scan showed recurrence in the lateral side of the original focus. The recurring foci were all treated with TACE and PMCT again. All patients remained alive during the follow-up period.

DISCUSSION

The mechanism of PMCT for HCC is based on the heating effect of microwaves and the sensitivity of the tumor to the heating action. The high temperature produced by the electrodes inserted into the tumor tissue results in the rapid coagulation and necrosis of the tumor tissue, thus achieving the goal of destroying the tumor. The area of PMCT is related to the diameter and number of the microwave electrode, the output power of the machine, coagulation time, and local blood circulation[7,10-13]. Increases in the diameter of the electrodes, the output power, and the coagulation time may enlarge the treatment areas of PMCT. However, increasing the diameter of the electrodes may also lead to increased rates of post-puncture hemorrhage, and an extension of the coagulation time may result in more pain for the patients. In practice, the diameter of the electrode and the output power of the microwave therapeutic machine are constant. Therefore, reducing the local blood supply is the only approach to achieving the goal of increasing the PMCT area.

It is well known that primary HCC possesses an abundant blood supply. TACE through the hepatic artery, especially the injection of iodine oil and gelatin sponge particles, blocks the artery supply to the tumor. The iodine oil can fill up the portal vein surrounding the tumor through the communication branches connecting the arterial and portal veins[14,15]. Thus, it can decrease the blood flow volume of the portal vein, and alleviate the adverse cooling effect induced by abundant blood flow[16,17]. In addition, TACE can result in ischemia of the tumor tissue and inflammatory edema, which accelerates tumor necrosis and enhances the coagulation effect of microwaves[18]. This forms the theoretical basis for the combined therapy of transcatheter hepatic artery chemoembolization with percutaneous microwave ablation for small HCC. Ishida *et al*[19] used hepatic artery embolization combined with a temporal block of the hepatic vein to reduce the segmental blood supply to the tumor. The therapeutic range of PMCT was increased; however, the procedures were too complicated to be practiced often.

Primary HCC is a malignancy that usually develops from liver cirrhosis, and is likely to have multiple foci. Although modern advanced diagnostic imaging techniques (spiral CT and MRI) can detect tumor nodules smaller than 1 cm, the detection rate is quite low. TACE therapy prior to PMCT enhances the efficacy of the latter. In addition, TACE can also help detect nodules that are undetectable by conventional imaging techniques, because digital subtraction angiography and the so-called iodine oil-CT are still the best methods for the detection of small HCC. Another advantage of TACE therapy prior to PMCT is that TACE can quickly and effectively control the foci that are inaccessible to PMCT.

Seki *et al*[20] treated 18 cases of small HCC of less than 3 cm using PMCT, and achieved complete necrosis in 17 of them. After a follow-up of 12-31 mo, all the patients were still alive. Lu *et al*[21] treated 67 cases of small HCC of less than 3 cm using the microwave therapy device produced by Nanjing Qinghai Company (Nanjing, China), and achieved a complete necrosis rate of 94% (63 cases). We used a combined therapy of transcatheter hepatic artery chemoembolization with PMCT for 35 cases of small HCC, and achieved complete necrosis in all cases. After a follow-up of 6-31 mo, only one patient had a recurrence at the original tumor site. Liang *et al*[22] analyzed the correlative factors for the prognosis of 288 HCC patients who received PMCT. They concluded that HCC patients with a single focus of a diameter $\leqslant$ 4 cm, and a liver function of Child-Pugh class A can have long-term survival after PMCT. Therefore, PMCT is ideal for small HCC. It can completely substitute for surgical resection, especially for elderly patients or those with a poor general condition or poor liver function[23].

Pathological examinations indicate that HCC is very likely to invade the capsule or extracapsular tissues[24,25]. Therefore, to achieve the goal of complete necrosis of tumor tissue and to increase its overall therapeutic efficacy, PMCT must not only destroy the tumor cells in the center of the tumor, but also destroy the adjacent non-cancerous hepatic parenchyma tissue. The microwave therapy device used in our study could produce coagulative tissue necrosis with a diameter of 4-5 cm under the conditions of an output power of 60-70 W, and a coagulation time of 15 min. By TACE, the area of tissue necrosis using a single needle electrode was even larger, completely covering a liver cancer nodule of $\leqslant$ 3 cm, with a tumor-free margin of 5 mm. Among our patients, there was one case of recurrence at the original tumor site, with incomplete liver necrosis of the surrounding non-cancerous liver parenchyma. This was probably due to the invasion of the tumor into the capsule or subcapsular tissues, or an insufficient coagulation time, which resulted in the incomplete killing of the tumor cells.

Compared with ultrasound-guided treatment, the

present study was carried out under guidance of CT and has multiple advantages[26,27]. It allows for determination of the precise locations of the foci, especially after TACE because iodine oil accumulation in the tumor foci makes the identification of their location and range easier. Additionally, when a plain CT scan is unable to clearly show the location of a tumor due to its small size, CT-guided puncturing of the focus can be performed based on the result of the MRI scan, thus reducing the "blindness" of the therapy. Finally, CT-guided liver puncturing has almost no "blind area", and is not affected by the adjacent organ full of gas.

Only mild adverse reactions were reported by our patients treated by PMCT. Local pain, during and after the treatment, was not very severe. No internal bleeding, subcapsular hematoma, biliary duct damage, or spreading of the tumor along the puncturing pathway were found. This suggests that PMCT is a safe therapy. Our results show that the combined TACE and PMCT for small HCC ($\leqslant$ 3 cm in diameter) is minimally invasive, simple and efficacious. Although further studies are needed, this combined therapy appears to be the first choice of treatment for small HCC that is unsuitable for surgical resection.

COMMENTS

Background

Surgical resection, transcatheter arterial chemoembolization (TACE), and percutaneous ethanol injection (PEI) are effective therapies for small hepatocellular carcinoma (HCC). However, for patients with poor liver functions, hepatectomy is not the first treatment choice. TACE is not effective for HCC with a poor blood supply, and PEI only yields incomplete necrosis of the tumor due to the uneven distribution of ethanol.

Research frontiers

TACE has the advantage of reducing the local blood supply of the tumor foci, resulting in tissue necrosis and inflammatory edema. Therefore, it can decrease the cooling effect of blood flow on the heating action of microwaves, and enhance the coagulating action of microwaves.

Innovations and breakthroughs

In the present study, the authors investigated the efficacy of the combined TACE and PMCT for small HCC.

Applications

The study shows that combined TACE and PMCT for treatment of small HCC ($\leqslant$ 3 cm in diameter) is minimally invasive, simple and efficacious. Although further studies are needed, this combined therapy appears to be the first choice of treatment for small HCC that is unsuitable for surgical resection.

Peer review

This is an interesting study which shows the advantages of combined TACE and PMCT for small HCC. It may provide useful information for us.

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CASE REPORT

Hepatitis B virus mutations potentially conferring adefovir/tenofovir resistance in treatment-naive patients

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Abstract

Anti-hepatitis B virus (HBV) therapy leads to the emergence of mutant viral strains during the treatment of chronic hepatitis B with nucleos(t)ides analogues. The existence of HBV variants with primary antiviral resistance may be important for treatment choice. We studied two patients with chronic HBV infection by sequencing the HBV polymerase gene. They had adefovir- and tenofovir-related mutations in the viral polymerase, although they had never been treated. These mutations were rtV214A/rtN238T in one patient and rtA194T in the other. Thus, mutations in untreated patients deserve cautious surveillance. These data indicate that mutations that can theoretically confer adefovir or tenofovir resistance may emerge in treatment-naive patients.

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Key words: Hepatitis B virus; Viral polymerase mutations; Treatment-naive patients

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INTRODUCTION

Hepatitis B virus (HBV) infection is a major public health problem, with approximately 350 million individuals chronically infected worldwide. Chronic HBV carriers are exposed to the risk of complications, including chronic hepatitis, cirrhosis and hepatocellular carcinoma. Up to one million people die every year from complications of HBV infection[1].

The discovery and clinical use of antiviral agents, targeting in particular the viral reverse transcriptase, have revolutionized therapy for patients chronically infected with HBV[2]. While each nucleotide or nucleoside produces efficient viral suppression, they induce only modest rates of HBV surface antigen (HBsAg) seroconversion and require long-term administration to control disease in patients. The need for long-term therapy necessitates drug safety and the ability to delay or manage the emergence of resistant HBV strains[3-5]. The clinical benefit of these therapies has been compromised by the emergence of resistant viral strains that carry specific mutations in the polymerase gene[6,7]. Elsewhere, mutations can be observed in treatment-naive patients, but very little is known about the frequency and impact

of these HBV variants[8-11].

In this context, different treatment options for the optimal management of chronic hepatitis B deserve to be mentioned. According to the European Association for the Study of the Liver, interferon alpha or nucleos(t)ides analogues can be used[12]. The indications for treatment, for patients with HBe antigen (HBeAg)-positive or -negative chronic HBV infection, are based on three criteria: serum HBV DNA > 2000 IU/mL, serum alanine aminotransferase (ALT) above the upper limit of normal, and liver biopsy showing at least grade A2 or stage F2 by METAVIR scoring. As a summary of recommendations, interferon can be proposed in patients with high baseline ALT (> 3 times upper limit of normal) and HBV DNA < 2×10^6 IU/mL at baseline. Interferon gives higher rates of HBeAg and HBsAg seroconversion but has frequent side effects. The second option is treatment with nucleos(t)ides analogues with potent antiviral efficacy, good tolerance but lower rates of HBe and HBs seroconversion, possibly indefinite duration of treatment, and risk of HBV resistance.

In the context of the introduction of HBV sequencing in the Virology Laboratory of Strasbourg University Hospital, with the aim to explore genotypic viral resistance, 14 untreated patients with chronic HBV infection were investigated for the HBV polymerase gene. Two of 14 patients showed, in the absence of treatment pressure, mutations theoretically linked to adefovir and tenofovir pressure.

CASE REPORT

In December 2005, a 50-year-old Vietnamese man was diagnosed with chronic HBV infection, without co-infection by hepatitis delta virus, hepatitis C virus (HCV) or human immunodeficiency virus (HIV). This diagnosis was established by exploring asthenia associated with arthralgia. As mentioned during the medical investigation when the patient was admitted to the University Hospital of Strasbourg, the infection was acquired by vertical transmission, which is common in Asia[13,5]. At the time of diagnosis, HBsAg and HBeAg were positive and anti-HBc antibodies were detected. The patient's ALT and aspartate aminotransferase (AST) values were seven times the upper limit of normal. The viral load was 1.08×10^8 IU/mL (COBAS TaqMan HBV test; Roche Diagnostics). In March 2006, a liver biopsy confirmed the diagnosis of chronic hepatitis B with moderate activity and extensive fibrosis (Metavir score A2F3). Sequencing of the HBV polymerase (TRUGENE® HBV genotyping kit; Siemens Medical Solutions Diagnostics, France) revealed two mutations potentially linked to adefovir resistance: rtV214A and rtN238T (confirmed by a second sequencing).

The second patient, a 35-year-old Moroccan man, was diagnosed with HBV infection in May 2003, without co-infection by hepatitis delta virus, HCV or HIV. He presented no past history of hospitalization, transfusion or drug addiction. HBsAg was positive, HBeAg was negative, and anti-HBc antibodies were detected in the absence of anti-HBs antibodies. The viral load was 43 200 copies/mL (Amplicor HBV test; Roche Diagnostics). ALT and AST values were normal. A liver biopsy performed in July 2003 confirmed the diagnosis of chronic hepatitis B (Metavir score A1F0). Therefore, a simple follow-up was proposed to the patient. In December 2005, sequencing of the viral genome revealed an rtA194T mutation of the polymerase (confirmed by a second sequencing), possibly linked to tenofovir resistance.

DISCUSSION

In these two cases, viral variants with mutations that confer potential adefovir or tenofovir resistance were discovered in patients who had never been treated.

The rate of selection of adefovir resistance is around 30% after 5 years of treatment. Adefovir resistance is associated with a primary mutation in the D domain at rtN236T. In addition, a number of other mutations have been detected that cluster into three distinct regions of the polymerase: the D and A domains (rtP237H, rtN238T/D, rtV84M and rtS85A); the B domain at rtA181T/V; and the C-D interdomain (rtV214A, rtQ215S). These mutations may be regarded as secondary resistance mutations, as they are associated only with very low-level resistance *in vitro*. These secondary mutations have also been detected in the absence of rtN236T (both alone and in combination) in patients who have either not responded or have had a virological breakthrough during adefovir treatment[6]. The rtN238T mutation may be involved in disruption of triphosphate binding in viral polymerase. However, other authors have suggested that background polymorphisms including rtV214A and rtN238T could exist without any impact on antiviral treatment failure[8].

Tenofovir resistance conferred by rtA194T in association with the changes that cause lamivudine resistance, i.e. rtL180M and rtM204V, has been observed in individuals who are co-infected with HBV and HIV-1[14,15]. However, the analysis reported by other authors has not provided a clear association between rtA194T and viral load rebound[4]. Thus, the potential impact of this mutation on tenofovir susceptibility deserves further study.

Two hypotheses come to mind. The first one is that the mutations appeared in the course of the chronic infection. The probability of selecting antiviral resistance is usually proportional to the intensity of selection pressure and the diversity of HBV quasispecies[6]. In our cases, treatment was not the means of selection pressure that allowed the mutated clones to take over. In our study, the pressure may correspond to the patients' immune system, or the mutant clones that bear the rtV214A, rtN238T or rtA194T mutations may confer a replication advantage on the wild-type virus. This hypothesis suggests that there is a natural polymorphism in the population with chronic hepatitis B, which might predispose to resistance to certain antiviral agents. The second hypothesis is that the patients may have been

infected with strains from other patients who had been treated with the corresponding nucleotide analogues.

Although the fact that two treatment-naive patients were infected by HBV strains with mutations in viral polymerase is interesting, two limitations have to be considered. First, since only 14 patients were studied, the prevalence of these changes in treatment-naive patients cannot be safely established. Large-scale investigations in HBV-infected patients, before any anti-HBV treatment**,** should be conducted in order to determine this prevalence. Second, the changes observed in positions 194, 214 and 238 of HBV polymerase do not represent well-established HBV resistance mutations[4,8]. Based on *in vitro* results, with controversial data reported for the rtA194T change by Delaney *et al*[4] and for rtV214A and rtN238T by Borroto-Esoda *et al*[8], the clinical significance of these mutants remains questionable.

In conclusion, our results concerning HBV mutations in treatment-naive patients that potentially confer resistance suggest the need for studies on large cohorts. By analyzing HBV sequences before antiviral therapy with analogues in treatment-naive patients, the clinical impact of pre-treatment mutations on the efficacy of antiviral therapy may be better characterized[16].

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CASE REPORT

Complete peritonectomy and intraperitoneal chemotherapy for recurrent rectal cancer with peritoneal metastasis

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Abstract

A 68-year-old man underwent laparoscopic low anterior resection for rectal carcinoma in December 2006. Nearly 19 mo after the operation, he developed recurrent rectal cancer with peritoneal metastasis. In September 2008, he subsequently underwent a laparotomy with a peritonectomy, omentectomy, splenectomy, and a Hartmann procedure. Hyperthermic intraperitoneal oxaliplatin 750 mg was administered. The patient was discharged with no postoperative complications and has been well with postoperative FOLFOX chemotherapy.

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Key words: Peritonectomy; Peritoneal metastasis; Recurrent rectal cancer

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Huh JW, Kim YJ, Kim HR. Complete peritonectomy and intraperitoneal chemotherapy for recurrent rectal cancer with peritoneal metastasis. *World J Gastroenterol* 2009; 15(6): 756-757 Available from: URL: http://www.wjgnet.com/1007-9327/15/756.asp DOI: http://dx.doi.org/10.3748/wjg.15.756

INTRODUCTION

Peritoneal carcinomatosis has a poor prognosis, and survival is generally less than 6 mo once diagnosed[1,2]. Recent advances in the treatment of this disease have resulted in improved, and even long-term, survival for select patients[3-5]. Optimal cytoreduction and adjuvant intraperitoneal chemotherapy have been credited with much of the recent success in the treatment of this cohort of patients. We would like to share our own experience with a complete peritonectomy and intraperitoneal chemotherapy for recurrent rectal cancer with peritoneal metastasis, and to review the relevant literature.

CASE REPORT

A 68-year-old man underwent laparoscopic low anterior resection (LAR) for upper rectal cancer. Histological examination revealed a poorly differentiated mucinous adenocarcinoma with a 4-cm distal margin and the TNM staging was T2N0M0. On routine follow-up, an anastomotic induration was felt on digital rectal examination 19 mo later, which was confirmed to be adenocarcinoma upon endoscopic biopsy. Abdominopelvic computed tomography and positron emission tomography showed multiple soft tissue infiltrations throughout the peritoneum. At laparotomy, a bulky mass around the previous rectal anastomosis and multiple peritoneal metastatic nodules were identified. A complete peritonectomy (Figure 1), complete omentectomy, splenectomy, and a Hartmann procedure were performed successfully with no visible gross disease at the completion of the procedure. Two catheters were inserted in the abdomen and secured: one on the left side aimed toward the pelvis and the other on the right directed up over the liver. The abdomen was closed, and the peritoneal cavity was filled with heated saline at 42℃. Continuous hyperthermic intraoperative intraperitoneal perfusion with 750 mg of heated oxaliplatin was then performed over 90 min. The operating time was 515 min with a moderate blood loss of 500 mL. Histopathology revealed a recurrent mucinous adenocarcinoma in the rectum with a clear resection margin and multiple metastatic nodules in the diaphragmatic, pelvic, and pericolic peritoneum and omentum. His postoperative course was uneventful, and he was discharged on the 25th postsurgical day. He had difficulty voiding, but this was managed conservatively. For 2 mo after the second

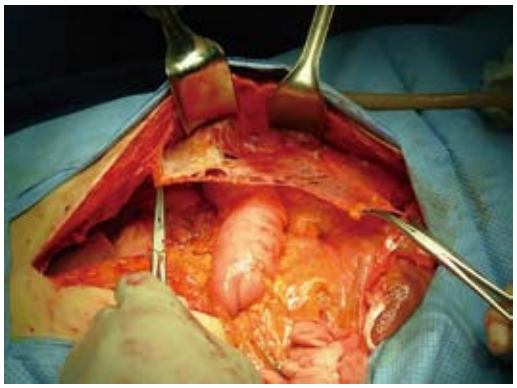

Figure 1 The peritonectomy.

operation, the patient has been well with postoperative FOLFOX chemotherapy.

DISCUSSION

Traditionally, there has been consensus in the oncology community that those patients with peritoneal carcinomatosis of colorectal origin were incurable. Neither systemic chemotherapy nor intraperitoneal chemotherapy alone had any significant impact on survival.

Recently, there has been increased interest in re-examining the management of peritoneal metastatic disease, and in utilizing cytoreductive surgery and hyperthermic intraperitoneal chemotherapy[6-9]. Aggressive cytoreductive surgery attempts to eradicate all residual tumor cells, or to significantly reduce the tumor burden, and the infusion of hyperthermic intraoperative intraperitoneal chemotherapy allows a favorable drug distribution to all surfaces at risk, without delay. A randomized trial by a Dutch group demonstrated superior survival with the combined approach over traditional 5-fluorouracil-based systemic chemotherapy, for peritoneal carcinomatosis of colorectal cancer[6]. Moreover, with proper patient selection, minimal morbidity can be achieved, with good overall survival and prolonged disease-free periods. The 11.1% rate of major perioperative complications and no perioperative mortality achieved in this cohort of patients compares favorably with the 27% quoted in the literature[10]. Elias *et al*[11] recently suggested that preoperative intraperitoneal chemohyperthermia with oxaliplatin is better tolerated than early postoperative intraperitoneal chemotherapy with mitomycin C and 5-FU, and is twice as efficient in curing peritoneal carcinomatosis.

This report describes our initial experience with peritonectomy and hyperthermic intraperitoneal chemotherapy in a patient with recurrent rectal cancer. This combined approach can be a feasible treatment option for the traditionally inoperable recurrent rectal cancer patient with peritoneal metastasis.

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CASE REPORT

Gastric pneumatosis intestinalis associated with malignancy: An unusual case report

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Abstract

Pneumatosis intestinalis (PI) is an uncommon disease defined as gas-filled cysts that are found in the wall of the gastrointestinal (GI) tract. The exact causes of PI are still unclear, but it may associated with coexisting diseases, such as some GI disorders, connective tissue disease, some medication and drugs, and rarely malignancy. The most common localization is the small intestine. Gastric PI secondary to malignancy has been rarely documented. We report on a 94-year-old man with gastric PI associated with inoperable adenocarcinoma localized in the duodenum. Following the gastrojejunostomy and choledochojejunostomy bypass, his general condition improved and PI disappeared, but he died due to poor performance status and malignancy 6 mo later. We suggest that in patients presenting with PI, malignancy should be considered in the differential diagnosis.

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Key words: Pneumatosis intestinalis; Malignancy; Adenocarcinoma

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INTRODUCTION

Pneumatosis intestinalis (PI) is a rare disorder characterized by multilocular gas-filled cysts localized in the submucosa and subserosa of the alimentary tract. It can occur in any part of the gastrointestinal tract (GI), from the esophagus to the rectum, but the small intestine is the most common localization[1-5]. PI may be primarily and secondarily associated with a coexisting disease. Eighty-five percent of patients have secondary PI[1,6,7]. Gastric PI, defined as air within the wall of the stomach, is an uncommon localization[8-12]. On the other hand, gastric PI, secondary to malignancy, has been very rarely reported in the literature[13]. We described here a case of gastric PI associated with adenocarcinoma localized in duodenum.

CASE REPORT

A 94-year-old man was admitted to our hospital with anorexia, nausea, vomiting, fatigue, constipation, weight loss, abdominal bloating and discomfort which started 1 mo previously, in May 2003. He had a prior history of peptic ulcer 15 years ago. He reported a history of upper GI bleeding in 1990. He had no the other systemic diseases, such as chronic obstructive lung diseases, connective tissue diseases, and no history of abdominal surgery. His family history was not contributory. On physical examination, he was dehydrated and afebrile. His blood pressure was 100/80 mm/Hg, pulse was 84/min, and heart sounds and jugular venous pressure were normal. No murmur was detected. Breath sounds were normal and there were no organomegaly or lympadenomegaly. Epigastric and periumblical sensitivity were noted. There was an abdominal distension and hypertympanism found on epigastric sites.

Initial laboratory results were as follows: blood urea nitrogen 23 mg/dL, creatinine 1.1 mg/dL, sodium 140 mEq/L, potassium 3.8 mEq/L, calcium 9.3 mg/dL, alanine aminotransferase 33 U/L, aspartate

Figure 1 Abdomino-pelvic CT scan showing big stomach and multilocular gas within the wall of the stomach (arrows).

Figure 2 Barium-enema study showing luminal obstruction of 80% in the bulber apex.

aminotransferase 27 U/L, γ- glutamyltransferase 428 U/L (N:7-49) alkaline phosphatase 317 U/L (N:38-155), lactate dehydrogenase 412 IU/L, glucose 105 mg/dL, erythrocyte sedimentation rate 40 mm/h, C-reactive protein 136.4 mg/L, total bilirubin 0.78 mg/dL, direct bilirubin 0.13 mg/dL, white blood cells (WBCs) 12400/mm^3, platelets 217000/mm^3, hematocrit 36.3%, mean corpuscular volume 84.9 fL, Fe 47 μ/dL, total iron binding capacity 214 μ/dL, and ferritin 55.4 ng/mL. Serum protein electrophoresis was normal. The patient's tumor marker profile was as follows: carbohydrate antigen 19-9 903.1 U/mL (0-37), carcinoembryonic antigen 12.3 ng/mL (0-3.4). Urine analysis revealed a urine specific gravity of 1010, protein negative, pH 5, and two WBCs per high-power field on urine sediment. Hepatitis B and C serology, and anti-HIV assays were negative. Fecal occult blood test was positive for the one stool sample. Other laboratory values were within normal limits.

The patient was treated with nasogastric intubation, parenteral fluids, ranitidine i.v. and broad-spectrum antibiotics. Because of elevated tumor markers, a diagnostic work-up for possible malignancy was initiated. The plain abdominal radiography (PAR) was normal. Abdominal ultrasonography (US) revealed sludge within the lumen of the gall bladder with findings of hydrops. The intrahepatic bile ducts were dilated (right, 5.3 mm; left, 4.5 mm). Abdomen and pelvic CT scans showed an enlarged stomach and multilocular gas-filled cysts localized in the wall of the stomach (Figure 1). The diagnosis of PI was made. Subsequent magnetic resonance cholangiopancreatography imaging demonstrated sludge with findings of hydrops and dilatation of intrahepatic bile ducts and choledochus. There was a calculius of 2 cm diameter at the neck of gall bladder.

A barium-enema study revealed luminal obstruction of 80% (Figure 2). As a result of these findings, gastroduodenoscopy and biopsy were performed. In the antrum, the ulcer had a 4-5 cm diameter, likely a malignant ulcer, and it was established that the lesion obstructed the lumen in the postbulbar duodenum. Malignancy was not detected in biopsy specimens. After the patient's general condition markedly improved, exploratory laporatomy was performed in the department of general surgery. An inoperable tumor originating from the duodenum was detected. In addition, the tumor was infiltrated to extra-hepatic bile ducts and vascular structures. Gastrojejunostomy and choledochojejunostomy were carried out. The histopathological examination of biopsy specimens revealed adenocarcinoma infiltration originating from the GI tract. As a result of his age and performance status, we decided to treat with supportive management. Following the surgical procedure, the gas-filled cysts disappeared and his symptoms improved. After 6 mo of follow-up, he died from the the influence of malignancy and poor general condition.

DISCUSSION

PI is an uncommon entity in which gas-filled lesions are accumulated within the GI wall. In 42% of the cases with PI, the small intestine is the most common and the large intestine (36%) the second most common localization; in 22% of patients both small and large intestine are affected[1,2,6]. On the other hand, gastric PI has been rarely documented[8-12]. It can be classified as either idiopathic or of primary unknown etiology (15%) or the secondary type (85%)[1,6,7]. Fifty-five percent of secondary causes are peptic ulcer related to pyloric obstruction, and 3.3% of cases are GI malignancy[2]. In our patient, the gastric secondary type of PI associated with GI malignancy was detected. Gastric PI related to malignancy has been documented extremely rarely[13].

Although the pathogenesis of PI is not well understood, many theories have been proposed. First, the mechanical theory suggests that gas under pressure diffuses into the bowel wall from the intestinal lumen or the pulmonary airway. This theory is probably related to PI caused by trauma, surgery, endoscopy and bowel obstruction. The bacterial theory proposes that bacteria enter the bowel wall and produce gas within the intestinal wall[3,14]. The pulmonary theory proposes that alveolar rupture results in the diffusion of air through the mediastinum into the retroperitoneal space, through the diaphragm, along major vessels in the mesentery, and into the bowel wall. It may explain the occurrence of PI in patients with chronic obstructive pulmonary disease. Finally, there is the mucosal damage theory. It suggests that gas under pressure is forced into the bowel wall

by mucosal disruption. PI associated with peptic ulcer disease, pyloric stenosis and malignancy may probably be explained by this theory[2,3].

PI is usually asymptomatic, but patients can have gastrointestinal symptoms variying in severity, such as abdominal discomfort, distension and pain, rectal bleeding, constipation, meteorism and weight loss. The symptoms are dependent on the localization of PI and on the presence or non-presence of underlying disease[2,3,6]. Our patient also presented with gastrointestinal symptoms and elevated tumor markers. After PI was detected, we initialy started a diagnostic work-up to exclude malignancy as a cause of PI. The work-up revealed an inoperable adenocarcinoma originating from the duodenum. After gastrojejunostomy and choledochojejunostomy, the gas-filled cysts disappeared and the patient improved.

In two-thirds of patients with PI, PAR shows characteristic changes in the bowel. The PAR with our patient was normal, but an abdomino-pelvic CT scan revealed PI. In the literature, additional diagnostic procedures, such as US, CT, MRI, endoscopy and barium contrast studies, have been documented[1,2,5]. There is no special treatment for PI. If underlying diseases are present, it is necessary to treat them[6]. Conservative therapy can be causal or symptomatic. Causal treatments consist of normobaric or hyperbaric oxygen therapy, antibiotic treatment, especially metronidazole, endoscopic puncture and cysts sclerotherapy[6,15-17]. The aim of these therapies is to suppress the etiological mechanisms. Surgical treatments should be avoided unless there are serious diseases, such as malignancy, metabolic acidosis, portal venous gas and severe inflammation[18].

This report constitutes a rare case of gastric PI caused by duodenal adenocarcinoma. In patients with PI, especially localized in the stomach, who have serious GI symptoms and elevated tumor markers, malignancy-induced PI should be considered in the differential diagnosis.

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CASE REPORT

Coexistence of tuberculous peritonitis and primary papillary serous carcinoma of the peritoneum: A case report and review of the literature

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Abstract

A major diagnostic challenge to the evaluation of an incomplete intestinal obstruction is to distinguish between infectious and malignant etiologies. We present a case of an elderly woman complaining of abdominal pain accompanied with nausea and vomiting, and failure to pass gas or stools. Anti-tuberculosis drugs were used to relieve her abdominal pain, and a needle biopsy of the peritoneal cavity showed evidence of primary papillary serous carcinoma of the peritoneum (PSCP). This is a rare description of tuberculosis in the setting of PSCP. This report illustrates the potential complex nature of malignancies, and emphasizes the need to consider coexistence of malignancy and infection in patients, especially in those with risk factors for malignancy who fail with antibiotic therapy.

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Key words: Primary papillary serous carcinoma; Peritoneum; Tuberculous peritonitis

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INTRODUCTION

A major diagnostic challenge to the evaluation of peritonitis is to determine whether it represents an infectious or malignant process. In the majority of cases, it is difficult to distinguish them from each other based on their clinical and radiographic data alone, and more invasive testing is usually required to reach a definitive diagnosis. In this report, we describe our experience with a case of peritonitis resulting from the coexistence of two distinct pathological processes.

CASE REPORT

A 59-year-old Chinese woman who was previously healthy presented to our clinic with a 2-mo history of intermittent abdominal pain accompanying general fatigue, subjective low-grade fever, weight loss and night sweats, which were not improved after treatment with over-the-counter antibiotics and traditional Chinese herbal drugs. Ten days before she visited our clinic, she felt her symptoms getting worse with nausea and vomiting, and weight loss of 10 kg within 2 mo. Thereafter, she failed to pass gas or stools which prompted her to seek medical care. She was admitted to 263 Hospital of PLA. A biochemical test showed 6500/mL white blood cells (WBCs), 78% N, 90 g/L hemoglobin, normal tumor markers (including carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 19-9, CA125, alpha fetoprotein (AFP), erythrocyte sedimentation rate (ESR) 148 mm/h), and 135.8 mg/L CRP. Abdominal plain film displayed ascites and incomplete intestinal obstruction. Chest X-ray showed a high density of WBCs in the right middle outer lobe and lymph node calcification in the right trachea bifurcation. Abdominal paracentesis was arranged for further evaluation. Routine test of ascites showed that yellow fluid was translucent, 95% mononuclear cells, 5% polynuclear cells, 6400/μL erythrocytes and 210/μL WBCs, and 1.010 gravity. Protein concentration was 44.7 g/L, glucose was 1.82 mmoL/L, lactate

dehydrogenase was 334 U/L, and adenosine deaminase was 15 U/L. Acid-fast bacillus could not be found in the fluid. Culture of bacteria was negative. Cytology of the fluid was also negative. CEA and AFP of ascites were normal. Ten days after treatment with antibiotics, her abdominal pain was aggravated, and she was transferred to our hospital.

The patient was a cotton spinner prior to retirement and underwent appendectomy 30 years ago. She denied any history of hepatitis or alcohol, tobacco or recreational drug use and had no knowledge of sick contacts. Physical examination showed symptoms of tenacious abdominal wall, deep and rebound tenderness, decreased intestinal sound, but no actual palpable abdominal mass. Liver and spleen were not palpable, shifting dullness was negative.

After admission, tuberculin was positive. Ultrasonography and pelvic cavity computed tomography (CT) showed ascites and mesentery thickening and peritonitis could not be excluded. Blood test results were 100/mL WBCs, 83.8% N, 85 g/L hemoglobin, and 545 000/mL platelets. Routine urine and feces tests were normal. Feces occult blood tests was negative (140 mm/h ESR, 218 mg/L CRP, and 29.6 g/L albumin). Two-thirds of anti-tuberculosis test parameters were positive. Tumor markers (CEA, CA199, CA125, and AFP) were normal. CA, CYFRA, FRER, and TSGF were 153 100 U/mL, 21-1 16.44 ng/mL, > 2000 ng/mL, and 121.45 U/mL, respectively. LTA was positive. Autoimmune antibodies were negative. Abdominal ultrasound showed small encapsulated effusion in abdominal and pelvic cavity. Parietal peritoneum was thick. Ultrasound of the vagina showed metratrophia with no occupying lesions in the pelvic cavity. Chest X-ray showed old tuberculous foci on both lungs and mediastinum. Due to the suspicious appearance of the lesion, a positron emission tomography (PET) scan was performed along with induced peritoneal cavity CT. Diagnostic percutaneous needle biopsy was discussed with the patient and her family, but she denied any invasive tests. PET scan showed an increased metabolic activity in peritoneum, mesentery, and areas of omentum with thick and diffusive lesser tubercles. Local intestinal adhesions or distensions showed incomplete intestinal obstruction.

She began to cough and had a low fever, but biochemical tests could not identify any infectious bacteria in her sputum and feces. Since the patient remained symptomatic, we decided to treat her disease with oral isoniazid, rifabutin, ethambutol, and pyrazinamide. During the first 6 wk, she noticed significant clinical improvements, such as reduction in abdominal pain and ascites, and resolution of tenacious abdominal wall. Body temperature became normal. However, 8 wk after antibiotic therapy, as well as enteral and parenteral nutrition, her condition began to deteriorate. In addition to CT and ultrasonography, the patient underwent percutaneous CT-guided biopsy of the thick peritoneum, which revealed primary papillary serous carcinoma of the peritoneum (PSCP). Although subsequent staging suggested that it was amenable to chemotherapy, the patient was too weak to tolerate the treatment. Finally, therapy failed to improve her condition and she developed liver and kidney failure.

DISCUSSION

The main differential diagnostic considerations of diffuse peritoneal involvement associated with peritonitis include infectious processes (mainly tuberculosis) and malignant neoplastic conditions. Peritoneal involvement is a rare form of abdominal tuberculosis. Peritoneal tuberculosis occurs predominantly in patients aged 20-40 years and is always secondary to other tuberculous lesions and appears to be more common in females than in males. Tuberculosis in females commonly reaches the peritoneum through tubal infection and attacks the tubes during the sexually active period of life[1]. Diagnosis of any extrapulmonary forms of tuberculosis is quite difficult. For example, difficult diagnosis of peritoneal tuberculosis is due to its non-specific clinical manifestations, such as weight loss, abdominal pain, fever, ascites, and vomiting[2-3]. Positive culture of 7.8% *Mycobacterium. tuberculosis* has been reported[4]. Although our patient denied previous medical history of pulmonary or extra-pulmonary tuberculosis, tuberculous infection was diagnosed based on her positive tuberculin test and chest X-ray scan.

PSCP is a rare malignancy that predominantly affects postmenopausal women[5]. Reports suggest that approximately 10% of women diagnosed with ovarian, endometrial or sigmoid carcinoma actually have PSCP[6-8]. Multicentric peritoneal involvement is typical, and omental involvement is particularly common. Extensive calcification of omental caking present in many cases is a useful CT finding for excluding mesothelioma. The absence of ovarian mass is critical for excluding metastatic papillary serous ovarian carcinoma, which otherwise has a similar appearance at CT and is histologically identical to its primary peritoneal counterpart[9]. Some reports indicate a poor prognosis for women with peritoneal carcinomatosis[10-13]. Patients suffering from PSCP usually complain of abdominal distention, pain or pressure, associated with ascites and gastrointestinal symptoms (loss of appetite, nausea, vomiting, and change in bowel habits)[14]. On physical examination, the most common finding is ascites. The clinical presentation is usually indistinguishable from advanced ovarian cancer. Reports suggest that approximately 10% of women diagnosed with ovarian carcinoma actually have PSCP[15].

In this case, a moderate amount of ascites located between intestinal canals was observed by ultrasonography, and a thickened intestinal wall and pronounced enhancement of peritoneum were seen at CT scanning. Most nodules coalesced to form large omental plaques (omental cakes) (Figure 1). The largest plaque was located in the left lower quadrant of the

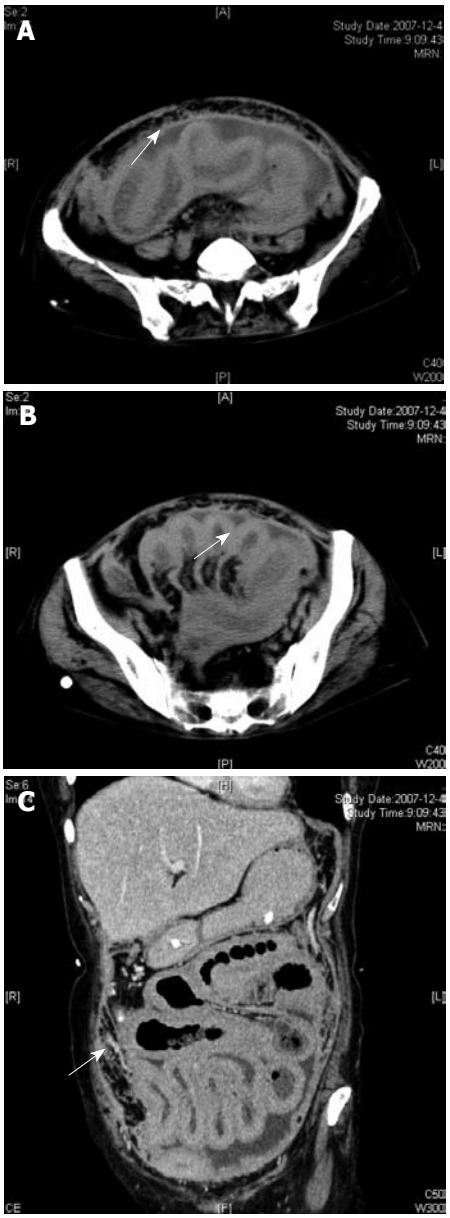

Figure 1 CT showing pronounced contrast enhancement of peritoneum and thickened intestinal wall. A: Most nodules coalesced to form large omental plaques (omental cakes); B: A moderate amount of ascites located between intestinal canals; C: No actual large abdominal masses.

abdomen, and extended to the pelvis, but did not involve the ovary. There was no calcification within the masses. The size of ovaries was normal. After treatment with anti-tuberculous drugs, the ascites decreased. Two months later, ascites stopped decreasing, suggesting that tissue biopsy is necessary to help its diagnosis. A delayed diagnosis or inadequate treatment, may promote progression to the malignant disease and the risk of life-threatening complications. To the best of our knowledge, this is the first report on the coexistence of PSCP and tuberculous peritonitis in humans.

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Meetings

Events Calendar 2009

January 12-15, 2009
Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

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

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

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

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

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

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

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

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

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

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

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

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

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

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

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

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

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

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

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

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

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

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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Instructions to authors

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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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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

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

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

Patent (list all authors)

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

Statistical data

Write as mean ± SD or mean ± SE.

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EDITORIAL

Anal transition zone in the surgical management of ulcerative colitis

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Abstract

Preservation of the anal transition zone has long been a significant source of controversy in the surgical management of ulcerative colitis. The two techniques for restorative proctocolectomy and ileal pouch anal anastomosis (RPC IPAA) in common practice are a stapled anastomosis and a handsewn anastomosis; these techniques differ in the amount of remaining rectal mucosa and therefore the presence of the anal transition zone following surgery. Each technique has advantages and disadvantages in long-term functional outcomes, operative and postoperative complications, and risk of neoplasia. Therefore, we propose a selective approach to performing a stapled RPC IPAA based on the presence of dysplasia in the preoperative endoscopic evaluation.

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Key words: Anal transition zone; Ileal pouch anal anastomosis; Restorative proctocolectomy; Ulcerative colitis

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INTRODUCTION

Restorative proctocolectomy and ileal pouch anal anastomosis (RPC IPAA) remain the standard of care in the surgical management of ulcerative colitis; however, controversy persists regarding preservation of the anal transition zone (ATZ). RPC IPAA is performed *via* either a stapled technique or a transanal mucosectomy and endoanal anastomosis, frequently referred to as a handsewn anastomosis. When the stapled technique is utilized, the ATZ is preserved; whereas a mucosectomy is usually performed from the level of the dentate line, therefore eliminating the ATZ and the proximal cuff of rectal epithelium. While the preservation of the ATZ has been shown to improve functional results and reduce operative time and complications, great debate persists regarding outcomes during recurrent or persistent disease and the theoretical risk of malignant transformation. Given the paucity of data in this realm, the long-term fate of the ATZ in the surgical management of ulcerative colitis has yet to be determined.

DEFINING THE ATZ

Defining the anatomy of the ATZ is difficult. Fenger first described the ATZ as "the zone interposed between uninterrupted crypt bearing colorectal-type mucosa above and uninterrupted squamous epithelium below"[1], which he characterized utilizing an Alcian dye technique. This technique is used for staining the ATZ macroscopically as the columnar epithelium stains dark blue, the squamous epithelium does not stain, and the ATZ stains pale blue. The Alcian dye technique delineates the margins of the ATZ from 6 mm below to 20 mm above the dentate line[1] with the median span from 0.73 to 0.89 cm[1,2]. Further studies by Thompson-Fawcett demonstrated that the Alcian dye technique overestimates the length of the ATZ when comparing this to computer mapping of the histological results[2]. Using computer histological mapping, the median upper and lower borders of the ATZ, measured from the

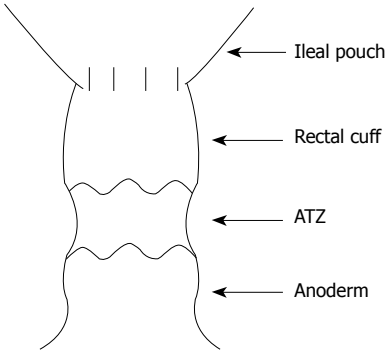

Figure 1 Retained rectal cuff after RPC IPAA.

lower margin of the internal sphincter, were 1.82 and 1.27 cm, respectively, with the dentate line measuring 1.05 cm by histology or 1.16 cm macroscopically above the lower margin of the internal sphincter. The median span was 0.45 cm *versus* 0.73 cm by the Alcian dye technique. This difference in length was accounted for by close examination of the histological specimens. The pale blue zone was due to staining of both superficial nuclei of thin squamous anoderm and the transitional epithelium that characterizes the ATZ. Fenger's technique for analyzing the ATZ did not exactly match the macroscopic Alcian blue specimens to the histological specimens, in comparison to Thompson-Fawcett's computer histological and Alcian dye mapping, and therefore likely overestimated the ATZ.

After careful measurements aided by computer mapping, the ATZ is now recognized to be smaller than previously considered. Significantly, columnar epithelium exists lower in the anorectal canal, and it is left behind when a stapled technique is utilized, which could be of consequence following RPC IPAA (Figure 1).

PRESERVATION OF THE ATZ: FUNCTIONAL OUTCOMES AND COMPLICATIONS

Duthie and Gairns in 1960 described a vast array of sensory nerve endings in the anal canal[3], which demonstrated sensitivity to temperature, touch, and pain, while the rectal mucosa lacked this innervation and sensation[3,4]. The rectum is able to sense distension, however, that results in a brief reflexive relaxation of the internal anal sphincter and contraction of the external anal sphincter, thus allowing the anal mucosa to sample the rectal contents. This sampling is thought to aid the ATZ in discrimination between gas, liquid, and solid stool. Anorectal sensation is abnormal in incontinent patients[5,6] and in patients following mucosectomy[7,8]. Thus, the extensive innervation of the ATZ is thought to play a very important role in maintaining fecal continence.

Following RPC IPAA *via* stapled or mucosectomy with endoanal anastomosis techniques, patients demonstrate decreased anal resting pressure (ARP)[9-13], thought to occur secondary to the extent of dissection, level of rectal transection, diameter of the stapling device, and anal retraction. However, ARP is greater following RPC IPAA stapled anastomoses than following mucosectomy with endoanal anastomoses[8,10,11,14,15]. Controversy exists regarding improvement of ARP over time when comparing the two surgical techniques. ARP has been demonstrated to return to a normal value by 12 mo after the operation only in RPC IPAA *via* a stapled technique[9], whereas Tuckson *et al* demonstrated no significant improvement in ARP 13 mo after either technique[10]. Both these studies analyzed a very small group of patients, with relatively short follow-up.

Functionally, preservation of the ATZ *via* RPC IPAA stapled anastomosis improves clinically relevant outcomes. Three prospective randomized controlled trials were performed in the early 1990s with limited numbers of patients (between 23 and 41 in each study). The results showed only limited benefit for stapled anastomosis with no worse outcomes[15-17]. More specifically, Luukkonen *et al* demonstrated no statistically significant differences between the only two functional parameters measured: number of nocturnal bowel movements and frequency of mucous leakage[17]. Choen *et al* also argued that many functional parameters showed no differences including frequency of bowel movements, number of nocturnal bowel movements, ability to delay defecation, and full continence[16]. However, Reilly *et al* demonstrated trends towards improved functional outcomes in all parameters measured: nocturnal seepage, daytime continence, ability to differentiate stool and flatus, and pad usage[15].

Other studies more clearly outline the functional benefit of stapled anastomoses. Remzi *et al* showed that patients with stapled anastomoses had better outcomes in every functional category measured, including statistically significant differences in incontinence, daytime and nighttime seepage, and pad usage[18]. Saigusa *et al* described an improved ability to discriminate between flatus and stool in the stapled *versus* handsewn group, 80% *versus* 33% ($P < 0.05$)[8]. Michelassi *et al* found that between 57% and 78% of patients were always able to delay a bowel movement until convenient; although this did not differ between the two groups[19]. These authors also demonstrated that stapled patients had improved rates of full continence, which persisted over time. Sagar *et al* studied stapled IPAA patients only for 12 mo postoperatively and compared parameters at 3 and 12 mo[9]. They showed that not only were functional outcomes better in the stapled IPAA group, but outcomes improved over time. Statistically significant improvements in the number of bowel movements ($P < 0.001$), number of nocturnal bowel movements ($P < 0.001$), frequency of loose stool consistency ($P < 0.001$), use of anti-diarrheal medication ($P < 0.01$), ability to defer defecation for more than 15 min ($P < 0.001$) and ability to discriminate flatus from stool ($P < 0.001$) were demonstrated. A single meta-analysis of 4183 patients (2699 handsewn and 1484 stapled IPAA) demonstrated no significant differences in the number of bowel movements, number of nocturnal bowel movements, daytime seepage, and use of anti-diarrheal

medication[14]. However, incontinence was more common in the handsewn group ($P = 0.009$) and the incidence of nocturnal seepage and nocturnal pad usage favored the stapled IPAA group ($P < 0.001$ and $P = 0.007$, respectively). Both groups were rated with a high quality of life with no statistically significant difference between the groups.

Early septic complications can occur following stapled or handsewn IPAA and may include anastomotic dehiscence with diffuse or localized sepsis, abscess, and fistula formation. These septic complications may require prolonged hospitalization, interventional procedures, reoperation, and pouch excision, therefore resulting in increased patient morbidity, prolonged recovery, and loss of bowel continuity. Risk factors for pelvic sepsis include ulcerative colitis, ulcerative colitis associated with toxic megacolon or fulminant colitis, male gender, and a handsewn anastomosis[20]. More than a two-fold increase in the rate of septic complications has been reported following handsewn *versus* stapled anastomoses[14,20,21], which results in increased rates of pouch failure requiring permanent diverting ileostomy[14,20,22] and more frequent pouch excision[14,21]. Pouch failure and pouch excision occur secondary to anastomotic dehiscence, poor functional results, pouchitis, perianal disease, and pouch leakage. Risk factors for pouch failure have been outlined and include handsewn anastomoses, anastomotic tension, use of diverting ileostomy, Crohn's disease, and postoperative anastomotic leak[22]. Further supporting the use of a stapled technique, when anastomotic leaks do occur, a better prognosis has been shown following a stapled anastomosis[20,22].

INFLAMMATION AND DYSPLASIA IN THE ATZ

It is well documented that chronic inflammation may lead to dysplasia and dysplasia may ultimately lead to neoplasia in patients with long-standing ulcerative colitis. The retained ATZ following stapled RPC IPAA is therefore at risk for chronic inflammation from recurrent or persistent disease, dysplasia, and possibly malignancy. The retained ATZ and potentially rectal mucosa is greater in length in the stapled anastomosis patient but can still be present in the handsewn patient due to the variation in location of the ATZ and incomplete transanal mucosectomy[2,23,24].

Inflammation within the ATZ is well documented. In early studies the incidence of endoscopic anal canal inflammation confirmed by biopsy was reported to be as high as 22% as estimated by Lavery *et al*, while the incidence of symptomatic inflammatory changes in the retained mucosa was 14.7%[25]. More recent prospective data document the incidence of inflammation following stapled RPC IPAA to be much higher: 4.6% with acute inflammation, 84.9% with chronic inflammation, and only 10.5% with normal mucosa[26]. The presence of inflammation, however, does not seem to negatively affect functional outcomes, as our group has reported in a large prospective study of 225 patients, with 96% of them reporting perfect fecal continence, 5.3% using protective pads, and 93.2% being able to delay a bowel movement for more than 30 min[26]. Patients with chronic inflammation of the ATZ still report better functional parameters when compared to handsewn control patients, including statistically significant improved flatus *versus* stool discrimination, decreased protective pad usage, and decreased dietary modification regarding meal timing[27]. In addition, patients with chronic inflammation of the retained ATZ more commonly reported an improved quality of life than prior to surgery when compared to handsewn patients ($P < 0.001$)[27].

Several studies have published their experience with postoperative serial endoscopic surveillance and the risk of dysplasia following preservation of the ATZ *via* a stapled RPC IPAA. The principal risk factor for developing dysplasia in the ATZ was the presence of dysplasia or cancer in the proctocolectomy specimen, independent of the location; no other factors including age, sex, or duration of disease, appear to increase this risk[28-31]. Ziv *et al* reported eight cases of dysplasia in 254 patients with a mean postoperative follow-up of 2.3 years; however, repeat biopsy revealed dysplasia in only two of these patients, who underwent a transanal mucosectomy without evidence of cancer in the final specimen[28]. Remzi *et al* analyzed a total of 178 patients with a mean follow-up of more than 10 years and identified dysplasia in only eight patients (4.5%) and no evidence of cancer[30]. No dysplasia was identified by our group in 242 patients with a mean follow-up of 56 mo[27]. This small disparity in incidence may be explained by variation in patient selection. In our practice, we do not offer a stapled RPC IPAA to patients with documented, and confirmed by two independent pathologists, colorectal dysplasia, irrespective of degree and location. Instead, these patients will undergo a transanal mucosectomy with a handsewn anastomosis[27]. We believe that the presence of dysplasia irrespective of degree and location is an indication of "mucosal instability." Since the primary risk factor for developing dysplasia in the ATZ is the presence of dysplasia or cancer in the surgical specimen, we perform a complete mucosectomy in this patient population. Complete absence of dysplasia or cancer in the ATZ in our series after 56 mo of follow-up, supports this approach.

Overall, dysplasia within the ATZ is uncommon and the risk of developing cancer following RPC IPAA is even more unlikely with only 19 reported cases in the literature[31] (Table 1). Even following a mucosectomy with endoanal anastomosis, islands of rectal mucosa probably exist at or below the dentate line in at least 20% of patients[23,24], These islands of rectal tissue may not be easily visualized during endoscopic biopsy, and dysplastic or malignant transformation may ultimately be difficult to detect. In fact, of the 19 reported cases of adenocarcinoma, 13 occurred in patients who underwent transanal mucosectomy. Furthermore, six of these patients developed cancer within the pouch itself, raising concern for metaplasia of the pouch as a method for

Table 1 Adenocarcinoma after RPC IPAA for UC[1]

Reference	Yr	RPC IPAA	Preoperative diagnosis	Pathological diagnosis	Yr to CA[2]	Site of carcinoma
Ravitch	1984	HS	NR	NR	NR	NR
Stern	1990	HS	Dysplasia	HGD rectum	3	Pouch
Puthu	1992	NR	NR	NR	6	NR
Rodriguez-Sanjuan	1995	HS	Dysplasia	HGD rectum	3.5	Pouch
Sequens	1997	Stapled	Carcinoma	CA rectum	1	ATZ
Vieth	1998	HS	Carcinoma	CA colon, multifocal dysplasia	2	Pouch
Iwama	2000	HS	UC	LGD	18	Anastomosis
Rotholtz	2001	Stapled	UC	HGD distal margin	7	ATZ
Heuschen	2001	HS	Dysplasia	CA colon	3	Pouch
Laureti	2002	HS	Carcinoma	CA anastomosis	2	Anastomosis
Hyman	2002	Stapled	Dysplasia	HGD distal margin & colon	5	Rectal stump
Baratsis	2002	Stapled	UC	CA cecum, multifocal dysplasia	2	ATZ
Bentrem	2003	HS	UC	CA colon, dysplasia	14	Pouch
Hassan	2003	HS	UC	UC	2	Pouch
Negi	2003	HS	Dysplasia	HGD	5	Rectal stump
Bell	2003	Stapled	Dysplasia	HGD colon	12	Anastomosis
Lee	2005	HS	Dysplasia	HGD rectum	2	Anastomosis
Lee	2005	HS	Dysplasia	CA rectum	6.5	Anastomosis
Lee	2005	HS	UC	UC	16	Rectal stump
Schaffzin	2005	HS	NR	NR	25	Pouch
Knupper	2006	Stapled	UC	NR	3	Pouch
Walker	2006	HS	NR	Dysplastic colon & rectum	17	Pouch
Das	2007	HS	UC	UC	25	ATZ
Ota	2007	HS	NR	UC	7	Rectal stump
Ruffolo	2007	Stapled	Carcinoma	CA colon at two sites	3	ATZ
Koh	2008	Stapled	NR	NR	14	Pouch inlet
Pedersen	2008	HS	HGD	CA colon, dysplastic colon	11	Rectal stump
Chia	2008	Stapled	UC	CA	3	ATZ

[1]Excludes articles not in English and one study using Cavitron Ultrasonic Surgical Aspirator technique for rectal mucosal stripping; [2]Yr to presentation of carcinoma after RPC IPAA; HS: Handsewn anastomosis; Stapled: Stapled anastomosis; NR: Not recorded or unknown; UC: Ulcerative colitis, HGD: High grade dysplasia; CA: Carcinoma; LGD: Low grade dysplasia.

malignant transformation. Yet, Swedish research has demonstrated that although the pouch itself may undergo metaplasia, there has been no progression to carcinoma; dysplasia occurred in less than 4.4% of patients; no high-grade was observed; and experienced pathologists did not even agree on the presence of dysplasia itself[32]. None of these patients had documented dysplasia or carcinoma in the surgical specimen preoperatively. When reviewing the cases of carcinoma following RPC IPAA, it is important to note that all but three patients had documented dysplasia or carcinoma within the original surgical specimen[31]. Given these data and the extensive evidence for the development of carcinoma in ulcerative colitis patients following dysplastic changes of the colon, we believe that it is appropriate to label dysplasia as a marker for "mucosal instability." Therefore, it is our belief that the presence of dysplasia or carcinoma, irrespective of the severity or location, should preclude a stapled RPC IPAA.

The presence of dysplasia or malignancy within the ATZ raises concern regarding treatment. Invasive malignancy requires a complete IPAA excision and end ileostomy. On the other hand, clear data regarding management of dysplastic changes within the ATZ do not exist. Dysplasia may even sometimes be self-limiting. Interestingly, several studies have shown regression of low-grade and even high-grade dysplasia to normal mucosa in some patients by serial biopsies[28,30], which is consistent with findings seen in the colon and rectum[33]. Most authors recommend completion mucosectomy with pouch advancement for high-grade dysplasia and for recurrent or persistently positive biopsies of low-grade dysplasia[28,30]. Dysplastic changes may be a marker for mucosal instability and the risk of developing carcinoma in this setting should be eradicated.

CONCLUSION

Preserving the ATZ offers improved long-term function, clinical outcomes, and decreased postoperative complications and pouch failure; however, ATZ preservation carries a small risk for developing dysplasia or malignancy. As a result of the risk of developing dysplasia or cancer, a selective approach to stapled RPC IPAA should be undertaken, based on the presence of dysplasia, irrespective of the location and severity. Stapled RPC IPAA, and therefore preservation of the ATZ, should be reserved for those patients in whom multiple preoperative endoscopic biopsies rule out dysplasia or carcinoma in the entire colon. Transanal mucosectomy and handsewn IPAA should be performed in patients with biopsy-proven dysplasia, irrespective of the location and severity. Due to the significant inter-observer discrepancies noted, the presence of dysplasia should be confirmed by two independent pathologists. If these resection guidelines are utilized, it is believed that cancer risk can be further reduced, especially if postoperative endoscopic surveillance is employed.

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Morphological, kinetic, membrane biochemical and genetic aspects of intestinal enteroplasticity

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Abstract

The process of intestinal adaptation ("enteroplasticity") is complex and multifaceted. Although a number of trophic nutrients and non-nutritive factors have been identified in animal studies, successful, reproducible clinical trials in humans are awaited. Understanding mechanisms underlying this adaptive process may direct research toward strategies that maximize intestinal function and impart a true clinical benefit to patients with short bowel syndrome, or to persons in whom nutrient absorption needs to be maximized. In this review, we consider the morphological, kinetic and membrane biochemical aspects of enteroplasticity, focus on the importance of nutritional factors, provide an overview of the many hormones that may alter the adaptive process, and consider some of the possible molecular profiles. While most of the data is derived from rodent studies, wherever possible, the results of human studies of intestinal enteroplasticity are provided.

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Key words: Diabetes; Diet; Hormonal regulation; Intestinal resection; Mechanisms; Morphology; Nutrient absorption; Short bowel syndrome; Signals

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INTRODUCTION

The intestine has an inherent ability to adapt morphologically and functionally in response to internal and external environmental stimuli. This process is called intestinal adaptation, or enteroplasticity. In fact, intestinal adaptation may be considered as a paradigm of gene-environment interactions. The array of phenotypic adaptations includes modification of brush border membrane (BBM) fluidity and permeability, as well as up- or down-regulation of carrier-mediated transport. Intestinal adaptation occurs following loss of a major portion of the small intestine (short bowel syndrome, SBS), following chronic ingestion of ethanol, following sublethal doses of abdominal irradiation, in diabetes, with aging, and with fasting and malnutrition[1-3].

Following intestinal resection, morphological and functional changes occur depending upon the extent and site of bowel removed, as well as external factors such as the lipid content of the diet (reviewed in Thiessen *et al*[4]). The increase in nutrient absorption from this process of enteroplasticity following resection compensates for the loss of mucosal absorptive surface area and minimizes the malabsorption that could otherwise occur. Therefore, intestinal adaptation has important implications in survival potential and welfare of the host[5]. In contrast, the adaptive process may be deleterious: for example, in diabetes this process enhances sugar and lipid uptake, exacerbating prevailing hyperglycemia, hyperlipidemia and obesity[6].

MECHANISMS

Mechanisms of intestinal adaptation occur at a variety of levels: physiological, cellular and molecular. Signals

of adaptation may relate to various hormone levels, transcription factors, ATP levels, or to changes in concentration of luminal solutes[3]. Signals for and mechanisms of the enteroplasticity process may be different for the jejunum and ileum, as well as in the intestinal crypt and villous tip, explaining site-specific alterations and differences between crypt and villous enterocytes[1-2].

Rodents are commonly used in well-characterized models for assessing the process of intestinal adaptation[7]. For example, following small bowel resection in the rat, the remnant intestinal mucosa undergoes compensatory alterations in an attempt to restore normal absorptive capacity[8]. Morphological and functional changes include increases in crypt depth and villous length, enterocyte proliferation, as well as increased electrolyte, glucose and amino acid uptake[7-8].

The adaptive process has been defined in terms of transport kinetics. Changes usually occur in the value of maximal transport rate (Vmax) rather than in Michaelis affinity (Km) constant of specific nutrient transporters (sugars and amino acids)[9-10]. Furthermore, there may be alterations in permeability coefficients of nutrients transported passively such as short-, medium- and long-chain fatty acids and cholesterol[1-2,11]. Increased Vmax results from either up-regulation of the total number of transporters per enterocyte, increased number of transporting mucosal cells, or increase in the intrinsic activity of the transporter[12-13]. Intestinal resection also selectively changes passive permeability properties of the BBM, as demonstrated by increased uptake of fatty acids, an increase that is not due simply to the changes in mucosal surface area or the effective resistance of the intestinal unstirred water layer (UWL)[14]. Indeed, this altered permeability is due to changes in the lipophilic properties of the BBM caused by variations in-lipid content of the BBM[15], which represents part of the adaptive process.

DIABETES AND INTESTINAL RESECTION AS EXAMPLES

Intestinal adaptation in the rodent model of chronic diabetes involves changes at the transcriptional as well as the post-transcriptional level, leading to increased Na^+-coupled sugar absorption[16]. After inducing acute hyperglycemia in rats, there is rapid up-regulation of glucose transport across the (enterocyte) basolateral membrane (BLM)[17]. In this model, both the vascular as well as luminal glucose infusion causes an increase in glucose transport capacity across the BLM[18]. No significant increase in BLM cytochalasin B binding or in GLUT2 protein abundance was observed, suggesting that there may be a post-translational event that increases the number of GLUT2 proteins available for transport, such as the movement of GLUT2 to the BLM from a preformed pool within the enterocyte. Alternatively, intrinsic activity of the transporter may be altered in the absence of changes in transporter protein abundance. Changes in intrinsic activity of glucose transporters have been observed with hyperglycemia[19], diabetes[20], low luminal glucose concentrations[12], and following activation of mitogen-activated protein kinase (MAPK) and P13K[13].

Following extensive intestinal resection, there is hyperplasia of the remaining small intestine, which is often accompanied by enhanced uptake of nutrients[21]. Alterations in the cell kinetics that result in modification of nutrition status may be specific or non-specific. Non-specific mechanisms involve alterations that result in changes in intestinal mucosal mass and/or villous surface area, leading to modifications in uptake of all nutrients, including those that are absorbed passively[22]. Specific mechanisms involve up- or down-regulation of transporters responsible for uptake of nutrients, such as sugars or amino acids[1-2].

THE IMPORTANCE OF MORPHOLOGY

The observation that morphological modifications may accompany intestinal adaptation in the rodent small bowel resection model was first made by Dowling and Booth[21]. The remaining intestine after resection is hyperplastic, with greater villous height and crypt depth, leading to enhanced mucosal surface area. While increased nutrient absorption is observed, the morphological changes do not necessarily solely explain alterations in nutrient uptake. For example, 1 wk after 80% small bowel resection, the remaining intestine increased its mass to 50%-70% of its pre-resection level, yet uptake of glucose increased only to approximately 33% of the pre-resection level[8].

It is clear that dynamic morphological parameters of the intestine may also adapt. For instance, crypt cell production rates or enterocyte migration rates along the villi change in some situations of intestinal adaptation[23]. It is important that morphological alterations be considered when estimating kinetic parameters of absorption. Morphological modifications such as blunting of mucosal growth or mucosal hyperplasia after intestinal resection are observed when dexamethasone is given subcutaneously[24]. Both transporter kinetics and dynamic morphological parameters are altered in the adaptive process, and the influence of resection on nutrient uptake is due to integration of these processes. This may be due to altered cell kinetics changing the population of enterocytes along the villus, thereby leading to variations in the number of cells with transporters, or activity of the transporters[25-26].

OTHER EXAMPLES

Many models of intestinal adaptation have been described: glucose uptake has been found to be increased during pregnancy[27], and lactation[28], with ingestion of a high carbohydrate diet[29], hyperglycemia[30], with diabetes[15], high alcohol intake[31] and after intestinal resection[32]. Glucose uptake is decreased with aging[33], external abdominal radiation[34], and with use of total parenteral nutrition[35]. Most transporters are up-regulated by levels of dietary substrate levels, and yet toxic substances and

essential amino acids have the opposite effect[7,35-37]. These examples illustrate the diversity and variability of this enteroplasticity process.

Increases in nutrient absorption have been documented[38-40] in humans following intestinal resection. The role of morphological changes in this process, however, has not been conclusively demonstrated. Remnant small bowel lengthening and dilatation has been noted in patients with SBS, suggesting morphological mechanisms in human intestinal adaptation[41]. However, the morphological adaptation typical in rodent models[21,42] (hypertrophy or hyperplasia) is not necessarily observed in the human adaptive response[43-44]. Several studies have shown no increase in villous height or crypt depth among patients who underwent intestinal resection, compared to healthy controls[39,45]. With the existence of various anatomical, physiological and biochemical differences between the human and rodent gastrointestinal tracts[46], and a conspicuous lack of comparable human studies, the clinical adequacy of the rat as a model of intestinal adaptation remains to be determined. Accordingly, caution must be used when attempting to extrapolate findings from rodent studies to the human population. Is there a better model? The neonatal piglet has been used in short bowel studies[47-49], and has been used to determine the effects of insulin-like growth factor-1 (IGF-l) and dietary manipulations[48,50]. The degree to which the results obtained using this model reflect human findings has yet to be determined, and the rodent remains a popular model for studies of intestinal adaptation.

DIETARY REGULATION

The topic of dietary regulation of intestinal gene expression has been reviewed[29,51]. Dietary constituents provide continual environmental signals that elicit expression of a host of genes that influence intestinal adaptation[52]. Every day, enterocytes are exposed to different nutrients that vary according to the nutrient intake of the host. For this reason, the intestine must be able to adapt to variations in the dietary load and composition[29,53]. The intestine, like many other biological and engineered systems, is quantitatively matched to prevailing peak loads with modest reserve capacities. Indeed, physiological capacities are optimal and most economical if they ascribe to the adage "enough, but not too much"[53]. Therefore, intestinal enzymes and transporters are characterized by a "safety factor", a parameter that represents the ratio of its capacity to the load placed on it[54]. The maintenance of this reserve capacity is biosynthetically costly, but is necessary given the unpredictable nature of dietary contents.

Let us consider the process of enteroplasticity and parenteral *vs* enteral nutrition, dietary lipids, carbohydrates, proteins and polyamines.

Parenteral vs enteral nutrition

In rodent models using total parenteral nutrition (TPN), small bowel atrophy is well characterized[55-57]. Not surprisingly, the presence of luminal nutrients also contributes greatly to enteroplasticity[58].

Dietary lipids

The dietary fat content influences the uptake of hexoses and lipids into rabbit jejunum following ileal resection[14]. In using a rat model of SBS, early feeding of a high-fat diet increased lipid absorptive capacity of the intestinal remnant[59]. A high-fat diet decreased mucosal mRNA levels of the lipid binding protein FAT/CD36, and decreased oleic acid uptake by isolated enterocytes. Mice that were chronically fed a diet enriched in sunflower oil had increased liver fatty acid binding protein (L-FABP) mRNA levels in the small intestine[60]. The effect was specific to this gene, as the intestinal fatty acid binding protein (I-FABP) was unaffected.

Not only the amount of fat, but also the type of dietary fat may influence intestinal function. Keelan *et al*[61] tested the hypothesis that intestinal morphology and uptake of nutrients after resection of the distal half of the small intestine of rats responds to alterations in dietary content of saturated (SFAs) and polyunsaturated (PUFAs) fatty acids. Adult female Sprague-Dawley rats were subjected to a sham operation or to surgical resection of the distal half of the small intestine. Animals were fed chow for 3 wk, then either chow or isocaloric semisynthetic SFA or PUFA diets for a further 2 wk. The *in vitro* jejunal uptake of glucose was twice as high in animals that had undergone resection and were fed SFAs than in those fed PUFAs. Perhaps SFAs are necessary in the diet to ensure that adequate adaptation takes place.

Thiesen and colleagues examined the effect of dietary lipids on lipid uptake in rats post-resection. Intestinal resection had no effect on mRNA expression of early response genes (ERGs), proglucagon or the ileal lipid binding protein (ILBP), but was associated with reduced jejunal mRNA for ornithine decarboxylase (ODC) and for L-FABP[62]. These resection-associated changes in gene expression were not linked with alterations in intestinal uptake of long-chain fatty acids or cholesterol. In animals undergoing intestinal resection and fed SFA, there was a reduction in jejunal proglucagon mRNA expression as compared to those animals fed chow or PUFA. ODC mRNA expression in the jejunum of resected animals was reduced. Thus, dietary lipids modify uptake of lipids in resected animals, and ODC and proglucagon may be involved in this adaptive response[63].

The way by which dietary lipids alter gene expression and consequently change membrane composition and/or nutrient transport may be through activation of peroxisome proliferator-activated receptors (PPARs), hepatic nuclear factor-4 (HNF-4), nuclear factor κB (NFKB), and sterol response element binding protein-lc (SREBP-1c)[52]. By binding to these transcriptional factors, dietary lipids affect the rate of transcription and consequently the protein synthesis of nutrient transporters[51,64]. PPARs belong to the super-family of receptors that include the glucocorticosteroid receptor[65].

When the locally acting glucocorticosteroid budesonide was administered concomitantly with an SFA diet, jejunal uptake of glucose was increased but ileal uptake of fructose was reduced[66].

It has been suggested that dietary lipids participate in signal transduction involving activation of second messengers, such as cAMP, Ca^{2+} and diacylglycerol, thereby changing the mRNA expression[67]. Studies with glycosphingolipid have revealed the importance of these lipids and their metabolites in signaling pathways *via* the tyrosine kinase-linked receptors. This is a signal system mediated by protein kinase C (PKC), MAPK, other kinases, as well as by cytosolic Ca^{2+} concentration[68]. Additional new signals involved in adaptive intestinal response 3 d after 50% intestinal resection have been identified by cDNA microarray analysis. These include proline-rich protein 2, involved in wound healing; glutathione reductase, a gene involved in intestinal apoptosis; NF-2 family members, also involved in apoptosis; etoposide-induced p53-mediated apoptosis; basic Kruppe-like factor, a transcription factor that activates the promoter for IGF-1; and prothymosin-α, involved in cell proliferation[69,70]. These observations of altered expression of signals are useful to generate hypotheses that can be tested in future studies to establish whether these signals represent a primary or a secondary event in enteroplasticity.

The glycospingolipid, phospholipid, cholesterol and fatty acid composition of plasma membranes may be modified in mammalian cells[71]. For example, Keelan *et al*[72] demonstrated that alterations in dietary fatty acid saturation influence intestinal BBM phospholipid fatty acid composition in rats. The investigators proposed that previously reported diet-associated changes in active and passive intestinal transport are due at least in part to these alterations in the fatty acid composition in BBM phospholipids. A diet enriched with SFA is associated with increases in the saturation of BBM phospholipid fatty acids, while a diet enriched with PUFA is associated with an increase in the unsaturation of BBM phospholipid fatty acids[1-2].

Meddings[73] compared *in vivo* membrane lipid permeability within the same intestinal region, under conditions where membrane physical properties were radically altered by feeding rats an inhibitor of cholesterol synthesis. Marked reductions in membrane fluidity were observed due to replacement of membrane cholesterol with its precursor, 7-dehydrocholesterol. Associated with these alterations was a pronounced reduction in membrane lipid permeability. Therefore, BBM membrane lipid permeability, *in vivo*, appears to be correlated with the physical properties of the bilayer.

The degree of fatty acid unsaturation or saturation, as well as the cholesterol and ganglioside/glycosphingolipid content, are factors that influence fluidity of the BBM[74-75]. Changes in fluidity of the BBM may alter passive permeation of molecules and nutrients through this barrier, as well as conformation of binding sites on transporter proteins such as SGLT1, GLUTS[71,76]. For example, alterations in BBM fluidity influence passive uptake of lipids, as well as carrier-mediated D-glucose uptake[76,77]. While enhancement of fluidity increases the uptake of lipids, fluidization of BBM from enterocytes located on the villous tip decreases uptake of D-glucose to levels seen in the BBM from enterocytes located in the crypts[78].

While altered membrane lipid composition may act in part by changing viscosity or fluidity of the BBM, it may also alter the microenvironment surrounding the transporter and thereby modify transporter activity. Two types of specialized microdomains in the BBM have been identified: lipid rafts and caveolae. These regions are important in signal transduction as well as lipid and protein trafficking[79-81]. Lipid rafts are enriched in SFAs, cholesterol and gangliosides[80-82].

Feeding rats a diet containing gangliosides increases jejunal glucose uptake[83]. Feeding them a ganglioside-rich diet increases ganglioside content and decreases cholesterol content in the intestinal mucosa, plasma, retina and brain[84]. Similar changes in lipid composition of intestinal microdomains, or lipid rafts, occur following ganglioside feeding[85]. Although SGLT1 has been localized to these microdomains in renal epithelial cells[86], it is not known if sugar transporters reside in intestinal BBM microdomains. If this is the case, local changes in membrane fatty acids may affect the activity of transporter by altering the configuration of the protein, potentially exposing or masking transporter binding sites and thereby modifying nutrient uptake. Gangliosides may also influence intestinal sugar transport *via* alterations on pro-inflammatory mediators, many of which are known to influence intestinal sugar transport[87-89]. For example, in rats challenged with lipopolysaccharide, ganglioside feeding reduced the production of intestinal platelet activating factor, PGE2 and LTB4, as well as plasma levels of IL-1β and TNF-α[90].

Dietary carbohydrates

Dietary carbohydrate may induce the intestinal adaptive response by increasing the abundance of hexose transporters to facilitate a higher rate of sugar absorption[9]. In a murine model, intestinal glucose uptake was directly correlated with dietary carbohydrate load[29,36,91]. The effect of dietary carbohydrate on nutrient transporter abundance has been reported in several animal models. For instance, abundance of SGLT-1 in BBM and GLUT2 in the BLM was elevated in animals fed a high carbohydrate diet; associated with this enhanced level of protein was an increase in glucose absorption[17,92-93]. The GLUT5 transporter abundance was also elevated with enhanced consumption of dietary fructose, leading to increased fructose uptake[77].

Initiation of dietary glucose-induced adaptive response occurs in the intestinal crypts, where transport capacities of nutrient transporters are programmed[91,36-37,93]. Utilizing a mouse model, diet from a high to a low carbohydrate regimen reduced the amount of glucose transporter, as estimated from density of phlorizin binding. Enterocytes may adapt to the high carbohydrate diet by increasing

crypt cell turnover rate, enhancing enterocyte migration rate, as well as by reprogramming the capability of nutrient transporters in the crypts to accommodate to the requirement for higher monosaccharide transporters[93]. Alteration in the density of glucose transporters was first observed in the crypt cells, and over a 3-d period was subsequently seen in the villous tip cells. Thus, crypt enterocytes respond to the high carbohydrate diet by increasing glucose transportation density. These cells then migrate up the villus over the next 3 d, contributing to the adaptation process enhancing glucose uptake.

Animals fed a glucose-enriched diet have increased glucose uptake, resulting from up-regulation of both BBM and BLM glucose transporters[17,92,94]. During early development, precocious introduction of dietary fructose causes enhanced expression of fructose transporters and fructose transport, without changing glucose uptake[93]. The substrates glucose and fructose specifically up-regulate their own transporters, SGLT-1 and GLUT5. In contrast, increases in essential amino acids or other substances that are potentially toxic at high levels (such as iron, calcium or phosphorous) are associated with no changes, or even reductions, in transport[35,95].

In many cases, other nutrients may be equal, or even more potent, inducers of the transporter than the transporter's specific substrate. For example, young animals fed a diet enriched with PUFA have a decline in glucose uptake, as compared to animals fed an SFA-enriched diet[66,96,97]. Similarly, Vine *et al*[98] studied the effect of varying fatty acids on the passive and active transport properties of rat jejunum and found that an SFA-enriched diet increased Na^+-dependent glucose uptake when compared to a diet enriched with n6 PUFA. In contrast, in aged rats, glucose uptake is increased by PUFA and not by SFA[33].

Dietary fiber also modulates intestinal nutrient uptake. For example, in dogs, a diet enriched with fermentable fiber increases glucose uptake and GLUT2 transporter abundance[99]. *In vitro* studies, in which rat intestinal tissue was incubated with B-glucan isolated from barley or oats, show reductions in uptake of stearic and linoleic acids (Drozdowski *et al*, 2005, unpublished observations). Furthermore, many studies have investigated the effect of TPN supplemented with short-chain fatty acids, the products of fiber fermentation. Increases in glucose uptake, GLUT2 mRNA and protein, and intestinal morphology were observed in normal rats as well as in rats following intestinal resection[100-103].

Dietary proteins

Dietary proteins also have an impact on intestinal morphology and active amino acid transport[37,104]. Both *in vitro*[105] and *in vivo*[104] rat experiments have shown that a high-protein diet increases amino acid uptake in the jejunum. Alteration in the amount of dietary protein induces reversible adaptation of non-essential amino acid transport rate[106]. Feeding a high-protein diet to mice induces a 77%-81% increment in uptake of non-essential amino acids[35], yet only a 32%-61% increase for essential amino acids. A protein-deficient regimen reduces uptake of non-essential amino acids, such as aspartate and proline, and maintains or increases uptake for essential amino acids and alanine.

Glutamine is a key metabolic fuel for enterocytes, mediating cellular nucleic acid synthesis and proliferation. Parenterally fed rats demonstrate decreased atrophy of the intestinal mucosa following glutamine supplementation[106]. Glutamine administration also normalizes the reduced levels of intestinal adaptation in rats receiving TPN following intestinal resection[107]. It is noteworthy that some studies of oral glutamine supplementation in the rat have failed to document more than temporary mucosal proliferation[108].

Other amino acids may inhibit intestinal adaptation. Sukhotnik *et al*[109] examined effects of parenteral administration of the nitric oxide precursor arginine to rats following 75% small bowel resection. Arginine supplementation was associated with lower cell proliferation and greater enterocyte apoptosis. Thus, the nature of the adaptive response depends upon the type of amino acid and the needs of the animal.

Polyamines

Polyamines are found in all eukaryotic cells[110], and they play an important role in growth and differentiation[111]. Polyamines are obtained either from the diet, or *via* synthesis from ornithine[112]. Luminal perfusions of polyamines rapidly (in less than 5 min) enhance intestinal glucose uptake in rats and increase BBM SGLT-1 protein[113].

Polyamine synthesis or uptake may be an important event that initiates adaptive hyperplasia observed in the intestinal remnant after partial small bowel resection. Enteral diets supplemented with ornithine alpha-ketoglutarate (OKG), a precursor for arginine, glutamine and polyamines, enhances intestinal adaptation in models of intestinal resection[114,115]. Indeed, studies by both Tappenden *et al*[116] and Thiesen *et al*[62] suggest that ODC, a key enzyme in polyamine synthesis, may mediate the adaptive process in rats that is stimulated by the administration of either glucocorticosteroids or short-chain fatty acids to rats following intestinal resection.

HORMONAL REGULATION

Glucocorticosteroids (GCs)

Numerous hormones modify the form and function of the intestine. It is not clear whether any of the dietary features that modify the intestinal adaptive process do so by way of hormonal alterations. In a model of extensive intestinal resection (50% enterectomy), the remaining proximal and distal intestinal remnants were adequate to assess the morphology and function at these sites[9,106]. The GC prednisone had no effect on intestinal uptake of glucose or fructose in these resected animals[62]. In contrast, the locally acting steroid budesonide increased by over 120% the value of the jejunal Vmax for the uptake of glucose, and increased by over 150% the ileal uptake of fructose. The protein abundance and mRNA

expression of SGLT-1, GLUTS, GLUT2 and Na^+/K^+ APTase $\alpha 1$ and $\beta 1$ did not explain this enhancing effect of budesonide on glucose and fructose uptake. Budesonide, prednisone and dexamethasone reduced jejunal expression of the early response gene c-jun. In resected animals, the abundance of the mRNA of ODC in the jejunum was reduced, and GCs reduced jejunal expression of mRNA for proglucagon. These data suggest that enhancing influence of GC on sugar uptake in resected animals may be achieved by post-translational processes involving signalling with c-jun, ODC and proglucagon, or other as yet unknown signals.

In contrast, the uptake of D-fructose by GLUT5 was similarly increased with budesonide and with prednisone. Increases in the uptake of fructose was not due to variations in weight of intestinal mucosa, food intake, or in GLUT5 protein or mRNA expression. There were no steroid-associated changes in mRNA expression of c-myc, c jun, c-fos, of proglucagon, or of selected cytokines. However, the abundance of ileal ODC mRNA was increased with prednisone. Giving post-weaning rats budesonide or prednisone in 4-wk doses equivalent to those used in clinical practice increases fructose but not glucose uptake. This enhanced uptake of fructose was likely regulated by post-translational processes[62].

Growth hormone (GH)

GH has been suggested as possessing pro-adaptive properties[117]. In rats and piglets, GH administration results in an increase in small bowel length and function per unit length[118]. Hypophysectomized rats undergo mucosal hypoplasia of the small bowel, as well as a reduced adaptive response following resection that is restored by GH[119]. In contrast, transgenic mice expressing elevated levels of GH experience hypertrophy of the small intestine[118]. IGF-1 expression in the small bowel is regulated by GH and is believed to induce enterotrophic effects following resection[120,121]. In a rat model of SBS, acute IGF-1 treatment of TPN-fed rats produced sustained jejunal hyperplasia, and facilitated weaning from parenteral to enteral nutrition[122]. GH administration to normal rats has been reported to have positive effects on mucosal growth and intestinal adaptation following massive resection[123], although contradictory data exist[124,125]. Human and rabbit studies have indicated that increased nutrient transporter activity devoid of morphological changes may be the method of GH-induced intestinal adaptation[126].

GH administration inhibits liberation of glutamine from muscle during catabolic states in humans[127]. This suggests a possible role for combining GH and glutamine to achieve optimal adaptation. Trials investigating any such synergism in the rat have yielded conflicting results. Some studies have failed to demonstrate an additive effect of GH and glutamine in the enhancement of post-resection intestinal adaptation[128], while others have documented a positive synergistic effect[129]. For example, GH has been shown to enhance the absorption of amino acids using *ex vivo* human BBM vesicles[129]. An intestinal mucosal GH receptor has been described in rats and humans[130], and GH promotes cell differentiation and clonal expansion of these differentiated cells[131].

Human studies have suggested that the efficacy of GH and/or glutamine therapy in the adaptive response of the small bowel may be based heavily upon the clinical status of the patient[132]. Evaluation of the effect of such may facilitate further understanding of the pathology and physiology of the bowel adaptation process, as well as more clearly defining positive predictive indicators of the bowel's ability to be rehabilitated. Existing human data indicate that administration of high concentrations of GH can actually increase patient morbidity and mortality[133].

In studies of home parenteral nutrition (HPN)-dependent patients with SBS, the use of high-dose recombinant human GH (0.4 mg/kg per day) in controlled[133,134] and uncontrolled studies[135] has led to variable results. Patients were given glutamine supplements by mouth or parenterally, and their diet was modified. In the randomized, placebo-controlled study of Scolapio *et al*[133], subjects ingested a standardized 1500 kcal/d diet, which is clearly different from the hyperphagic diet consumed by many SBS patients[136], and which may contribute to the physiological adaptation that occurs in the remaining intestine after extensive resection. It is unclear whether glutamine is beneficial for the adaptive response in humans. In rat models of SBS, it is unclear whether glutamine supplementation is efficacious for the adaptive process[137-138]. Furthermore, both a hyperphagic diet and absence of malnutrition are needed for humans to achieve optimal intestinal adaptation[41,139].

When HPN-dependent patients with SBS were provided a usual *ad libitum* hyperphagic diet, and given low doses of GH (0.05 mg/kg per day) for 3 wk, there was significant improvement in intestinal absorption of energy (15% ± 5%), nitrogen (14% ± 6%) and carbohydrate (10% ± 4%)[140]. Increased food absorption represented 37% ± 16% of total parenteral energy delivery. Body weight, lean body mass, D-xylose absorption, IGF-1, and IGF binding protein 3 increased, whereas uptake of GH binding protein decreased. During treatment with GH, improvement in net intestinal absorption compared with placebo was 427 ± 87 kcal/d, representing 19% ± 8% of the total energy expenditure required to obtain energy balance equilibrium in patients with SBS[136].

From a review of literature in this area, Matarese *et al*[140] noted that there were differences in gastrointestinal (GI) anatomy, dietary compliance, nutritional status, presence of mucosal disease, and diagnosis both within and between studies. These authors concluded that "administering recombinant human growth hormone alone or together with glutamine with or without a modified diet may be of benefit when the appropriate patients are selected for treatment".

IGF-1

IGF-1 proved to be efficient in increasing intestinal adaptation following resection in rats. IGF-l treatment

following 70% jejuno-ileal resection attenuated fat and amino acid malabsorption[141] and increased total gut weight by up to 21%. The IGF-1 receptor was increased in the jejunum and colon due to resection. Resection also increased circulating IGF-binding proteins (IGFBPs). IGF-1 treatment had no effect on IGF-1 mRNA or IGF-1 receptor density, but increased IGFBP-5 in the jejunum[142]. This increase in IGFBP-5 was correlated with jejunal growth after IGF-1 treatment[142].

IGF-1 treatment in resected rats significantly increased jejunal mucosal mass by 20% and mucosal concentrations of protein and DNA by 36 and 33%, respectively, above the response to resection alone[143]. These changes reflected an increase in enterocyte proliferation and an expansion of the proliferative compartment in the crypt. No further decrease in enterocyte apoptosis, or increase in enterocyte migration[144].

IGF-1 treatment may also facilitate weaning from parenteral to enteral nutrition. After 60% jejunoileal resection plus cecectomy, rats treated with recombinant human IGF-1 (3 mg/kg body weight per day) or control vehicle were maintained exclusively with TPN for 4 d, and were then transitioned to oral feeding. TPN and IGF-1 were stopped 7 d after resection, and rats were maintained with oral feeding for 10 more days. Acute IGF-1 treatment induced sustained jejunal hyperplasia, as suggested from the presence of greater concentrations of both jejunal mucosal protein and DNA, and was associated with maintenance of a greater body weight and serum IGF-1 concentrations[122].

Using male transgenic mice with targeted smooth muscle IGF-1 over-expression[145], these as well as non-transgenic littermates underwent 50% proximal small bowel resection. Growth factor over-expression led to a unique mucosal response characterized by a persistent increase in remnant intestinal length and an increase in mucosal surface area. Therefore, IGF-1 signaling from within the muscle layer may be important in resection-induced intestinal adaptation. In summary, IGF-1 shows promise as a hormone which may prove to be of clinical significance in nutritional regulation and the modification of intestinal absorption in the short and long term[138].

Epidermal growth factor (EGF)

EGF up-regulates intestinal nutrient transport[146]. This effect is mediated by PKC and P13K[147], and involves redistribution of SGLT-1 from microsomal pools to the BBM[148,149]. After massive intestinal resection, endogenous EGF is increased in saliva and is decreased in urine[150]. EGF stimulates intestinal adaptation after intestinal resection: the BBM surface area and total absorptive area increased until day 10, and EGF treatment induced a further increase in BBM surface area[151]. In a study by O'Brien and colleagues[152], mice underwent 50% small bowel resection or sham operation, and were then given orally an EGF receptor (EGFR) inhibitor (ZD1839, 50 mg/kg per day) or control vehicle for 3 d. ZD1839 prevented EGFR activation, as well as the normal postresection increases in ileal wet weight, villus height, and crypt depth. Enterocyte proliferation was reduced two-fold in the resection group by ZD1839. These results more directly confirm the requirement of a functional EGFR as a mediator of postresection adaptation response. Previous work has demonstrated that the EGFR is predominantly located on the BLM of enterocytes[153], but after small bowel resection the EGFR shows redistribution from the BLM to the BBM, with no change in the total amount of EGFR[154]. It is not known how this redistribution occurs. This is an important point, since modification of this process may represent a useful means to accelerate the intestinal adaptive process.

Laser capture microdissection (LCM) microscopy was used to elucidate the specific cellular compartment(s) responsible for postresection changes in EGFR expression[155]. Mice underwent a 50% proximal resection or sham operation, and after 3 d, frozen sections were taken from the remnant ileum. Individual cells from the villi, crypt, muscularis and mesenchymal compartments were isolated. EGFR mRNA expression for each cell compartment was quantified using real-time reverse transcriptase polymerase chain reaction (RT-PCR). EGFR expression was increased two-fold in the crypts after resection. This supports the hypothesis that EGFR signaling is crucial for the mitogenic stimulus for adaptation. The additional finding of increased EGFR expression in the muscular compartment is novel and may imply a role for EGFR in the muscular hyperplasia seen after massive small bowel resection. As noted previously, it is of interest that the muscle layer also appears to play a role in the adaptive response to IGF-1[155].

Treatment of resected rats with EGF has been studied: male juvenile rats underwent either transection or ileocecal resection leaving a 20-cm jejunal remnant[156]. Resected animals were treated orally with placebo or recombinant human EGF. Resected EGF-treated animals lost significantly less weight than those in the transection group, absorbed significantly more 3-0 methylglucose, and had reduced intestinal permeability as determined by the lactulose/mannitol ratio. Work by Chung *et al*[149] using rabbits showed that intestinal resection altered SGLT-1 mRNA and protein expression along the crypt-villous axis, with expression being highest in the mid-villous region. Oral EGF normalized SGLT-1 expression, resulting in a gradient of increasing expression from the base of the villous to the villous tip.

Nakai and colleagues[157] investigated the role of EGF in stimulating intestinal adaptation following small bowel transplantation. Treatment of rats with EGF (intraperitoneally for 3 d) following intestinal transplantation resulted in increased glucose absorption, SGLT-1 abundance and villous height and crypt depth in the graft. Clearly, there are sufficient animal data to support studies of the potential pro-adaptive role of EGF in humans.

Keratinocyte growth factor (KGF)

In a study by Yang *et al*[158], adult C57BL/6J mice were randomized to 55% mid-small bowel resection, resection with KGF administration, or a sham-operated (control)

group, and were killed at day 7. Ussing chamber studies showed that KGF increased net transepithelial absorption of 3-0-methyl glucose as well as sodium-coupled alanine absorption, but had no effect on epithelial permeability barrier function. Epithelial cells were separated along the crypt-villous axis with LCM, and epithelial KGF receptor (KGFR) mRNA abundance was studied using real time RT-PCR. KGF up-regulated KGFR mRNA abundance, predominately in the crypt and the lower portion of the villus.

Leptin and ghrelin

Leptin plays an important role in the regulation of body fat and satiety (reviewed in Jequier[159]). Leptin reduces food intake[160] and leptin-deficient mice develop obesity[161]. Leptin may be a potential growth factor for the normal rat small intestine. The effect of 14 d of parenteral leptin administration (2 μg/kg per day) to rats following 80% small bowel resection was studied. Leptin was associated with a 44% increase in galactose absorption and a 14% increase in GLUT-5 abundance, but with no change in DNA content or in SGLT abundance. These findings suggest that leptin may potentially be clinically useful in patients with impaired intestinal function[162].

Ghrelin is a gastric hormone that is released in response to enteral nutrients. It has an opposite effect when compared to leptin, as it stimulates food intake[163]. The role of ghrelin in intestinal adaptation is unknown.

Glucagon-like peptide 2 (GLP-2)

Animal studies have demonstrated a potential role for GLP-2 in the adaptive response following intestinal resection[143]. Plasma GLP-2 levels rise following intestinal resection in rats[164-166]. In a study by Dahly *et al*[143], rats were subjected to 70% mid-jejunoileal resection or ileal transection, and were maintained with TPN or oral feeding. Resection-induced adaptive growth in TPN- and orally-fed rats was associated with a significant positive correlation between increases in plasma bioactive GLP-2 and proglucagon mRNA abundance in the colon of TPN-fed rats and in the ileum of orally fed rats. While these increases were transient in the TPN-fed group, luminal nutrients produced a sustained increase detected at 3 and 7 d post-resection. These data support a significant role for endogenous GLP-2 in the adaptive response to mid-small bowel resection in both TPN and orally fed rats[167].

Correlations between post-resection GLP-2 levels, morphological indices, crypt cell proliferation rates and nutrient absorption have been made[168]: an inverse correlation was found between post-prandial GLP-2 levels and fat or protein absorption, as assessed by a 48-h balance study. These results, along with data obtained from studies showing that GLP-2 immunoneutralization inhibits post-resection adaptation[169], further implicate GLP-2 as a post-resection mediator of adaptation.

GLP-2 administration in rats increases the adaptive response to massive intestinal resection[170]. In this study, Sprague-Dawley rats were divided into two groups, one with a 75% mid-jejunum-ileum resection, and a second sham operated group. Animals were treated with 0.1 pg/g GLP-2 analog (protease resistant human GLP-2) or placebo given subcutaneously twice daily for 21 d. The groups were compared measuring the total weight of the rats, and mucosal mass per centimeter. Administration of GLP-2 or its analogs was associated with an increase of the mucosal mass in the proximal jejunum and terminal ileum. While resection reduced D-xylose excretion, GLP-2 restored D-xylose excretion to levels above control values within 21 d of administration. This indicates that GLP-2 has a positive effect on intestinal morphology and absorptive function following resection.

Martin *et al*[170] investigated the effects of GLP-2 in a TPN-supported model of experimental SBS. Juvenile Sprague-Dawley rats underwent a 90% small intestinal resection and were randomized to three groups: enteral diet and intravenous saline infusion, TPN only, or TPN + 10 μg/kg per hour GLP-2. TPN plus GLP-2 treatment resulted in increased bowel and body weight, villous height, intestinal mucosal surface area, and crypt cell proliferation. GLP-2 reduced the lactulose-mannitol ratio, indicating that GLP-2 lowered intestinal permeability when compared with the TPN alone. GLP-2 increased serum GLP-2 levels as well as intestinal SGLT-1 protein abundance compared with either TPN or enteral groups. This study demonstrates that GLP-2 is capable of stimulating intestinal adaptation in the absence of enteral feeding.

Since a number of hormones and growth factors have been shown to influence intestinal function, Washizawa *et al*[171] compared the effects of GLP-2, GH and KGF on markers of gut adaptation following massive small bowel resection. KGF increased goblet cell numbers and TTF3, a cytoprotective trefoil peptide, in the small bowel and the colon. While both GH and KGF increased colonic mucosal growth, GLP-2 exerted superior trophic effects on jejunal growth. GLP-2 also increased the glutathione/glutathione disulfide ratio, resulting in improved mucosal glutathione redox status throughout the bowel. Because of the differential effects of GLP-2, GH and KGF on gut adaptation following massive small bowel resection, the authors conclude that a combination of these agents may be most beneficial.

A pilot study to determine efficacy of GLP-2 in patients with SBS has been completed. A non-placebo controlled study was conducted in eight patients with SBS with an end-enterostomy type of anastomosis (six had Crohn's disease and four were not receiving HPN)[172]. Treatment with GLP-2 (400 μg subcutaneously twice a day for 35 d) increased intestinal absorption of energy, body weight, and lean body mass. Crypt depth and villous height were also increased in five and six patients, respectively.

A review by Jeppesen[173] on the role of GLP-2 in treatment of SBS concludes that: "Currently, hormonal therapy in short-bowel patients should be considered experimental and it is only recommended in research

studies"[11]. The optimal duration and concentration requirements for GLP-2 to induce beneficial effects on intestinal secretion, motility, morphology and most importantly absorption, are not known. Optimal dosage and administration of this new treatment to short-bowel patients may eventually result in long-term improvements in nutritional status and independence of parenteral nutrition in a larger fraction of short-bowel patients.

OTHER POSSIBLE SIGNALS OF INTESTINAL ADAPTATION

Dodson *et al*[174] identified three subsets of genes that were up-regulated by constructing a cDNA library from the remnant ileum of resected rats. This library was screened, and subtractive hybridization was used to identify genes that were induced following resection. These included genes involved with regulating the absorption and metabolism of nutrients. For example, L-FABP, apolipoprotein A-IV, cellular retinal binding protein II and ileal lipid binding protein were identified as genes that were induced following 70% intestinal resection in rats. Genes involved in cell cycle regulation were also identified. For example, phosphorylation and dephosphorylation are important regulators of the cell cycle, and PP1S, a subunit of a serine/threonine phosphatase was indeed up-regulated. Grp78, a member of the heat shock protein family was also increased. Grp78 resides in the endoplasmic reticulum and acts as a chaperone during protein assembly and transport. It may also have a protective role, and prevent apoptosis as a way of promoting the proliferative response following intestinal resection[175-176].

Rubin *et al*[177] further characterized the molecular and cellular mechanisms following 70% resection in rats. An immediate early gene, PC4/TIS7, was markedly increased soon after resection, with levels returning back to normal by 1 wk post-resection. Although the function of this protein is unknown, it may be related to cytodifferentiation as it is expressed only in the villus and not in the crypts.

Erwin *et al*[70] used cDNA microarrays to gain insight into the mechanism of intestinal adaptation. Mice underwent a 50% intestinal resection, and 3 d afterwards RNA was extracted from the remnant ileum. Multiple genes were induced, and fall into four categories: (1) apoptosis, DNA synthesis, repair and recombination (ten genes); (2) oncogenes, tumor suppressors, cell cycle regulators (three genes); (3) stress response, ion channels and transport (four genes); (4) transcription factors and general DNA-binding proteins (one gene).

Many of the genes (ODC, c-neu, glucose-related protein, IGFBP-4) that were identified agreed with the results of other studies of intestinal resection. For example, ODC was increased in this study, and this agrees with previous findings that showed ODC to be involved in the adaptive process[60,116,178]. Some new factors were also identified including glutathione reductase (involved in apoptosis), basic Kruppel-like factor (transcriptional regulator that activates the IGF promoter), prothymosin-α (associated with increased cell proliferation), and eteposide-induced p53-responsive mRNA (stress response protein involved in p53 mediated apoptosis).

Stern *et al*[69] performed a similar analysis of gene expression following 50% intestinal resection in rats. The gene with the largest increase was identified as Sprr2, a novel gene not previously known to be involved in intestinal adaptation. EGF administration post-resection further increased sprr2 expression, and enhanced the adaptive response. This protein plays a role in terminal differentiation of stratified squamous epithelium. Its role in the intestinal epithelium is unclear and warrants further investigation.

Finally, a variety of other signals have been described as possibly playing a role in the process of intestinal adaptation. These include prostanoids[179], uncoupling proteins[180], PPAR-α, transforming growth factor-α[181], SPARC (secreted protein, acidic and rich in cysteine[182], Bcl-2[183], endothelin-1[184], erythropoietin[185], the GATA family of zinc finger transcription factors[186], hepatocyte growth factor[187], the ERGs[188], PC4/TIS7[177] and epimorphin[189]. Augmented Wnt signaling has been shown to enhance the adaptive response to massive small bowel resection[190]. Several of these signals may be useful to modify in a clinical setting to enhance the intestinal adaptive response.

Microarray technology is a powerful tool that is constantly developing into a more sophisticated technique of identifying novel genes involved in physiological processes. Intestinal adaptation awaits further characterization by hypothesis-testing studies. From the information that is available at this time, it is clear that genes regulating the cell cycle, proliferation, differentiation and apoptosis are important components of the adaptive process, leading to enteroplasticity.

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REVIEW

Epidemiology and gene markers of ulcerative colitis in the Chinese

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Abstract

Inflammatory bowel disease (IBD) includes two similar yet distinct conditions called ulcerative colitis (UC) and Crohn's disease (CD). These diseases affect the digestive system and cause the inflammation of intestinal tissue, form sores and bleed easily. Most children with IBD are diagnosed in late childhood and adolescence. However, both UC and CD have been reported as early as in infancy. Most information pertaining to the epidemiology of IBD is based upon adult studies. Symptoms include abdominal pain, cramping, fatigue and diarrhea. Genetic factors play a significant role in determining IBD susceptibility. Epidemiological data support a genetic contribution to the pathogenesis of IBD. Recently, numerous new genes have been identified as being involved in the genetic susceptibility to IBD: *TNF-308A*, *CARD15* (*NOD2*), *MIF-173*, N-acetyltransferase 2 (*NAT2*), *NKG2D* (natural killer cell 2D), *STAT6* (signal transducer and activator of transcription 6), *CTLA-4* (cytotoxic T lymphocyte antigen-4), *MICA-MICB* (major histocompatibility complex A and B), *HLA-DRB1*, *HLA class-Ⅱ*, *IL-18*, *IL-4*, *MICA-A5*, *CD14*, *TLR4*, *Fas-670*, *p53* and *NF-κB*. The characterization of these novel genes has the potential to identify therapeutic agents and aid clinical assessment of phenotype and prognosis in patients with IBD (UC and CD).

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Key words: Genetic; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Epidemiology; Susceptibility; Gene

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INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are the two primary types of inflammatory bowel disease (IBD). These two diseases have many similarities and sometimes are difficult to distinguish from each other. However, there are several differences. UC is an inflammatory destructive disease of the large intestine characterized by motility and secretion disorders. Inflammation usually occurs in the rectum and lower part of the colon, but it may affect the entire colon. UC rarely affects the small intestine except for the end section, called the terminal ileum. UC may also be called colitis or proctitis. Inflammation frequently makes the colon empty, causing diarrhea. Ulcers formed in places where the inflammation has killed the colon cells cause, bleeding and pus discharge. UC is an IBD that causes inflammation in the small intestine and colon. UC is difficult to diagnose because its symptoms are similar to other intestinal disorders and another type of IBD called CD. CD differs from UC because it causes deeper inflammation within the intestinal wall. CD usually occurs in the small intestine, although it can also occur in the mouth, esophagus, stomach, duodenum, large intestine, appendix, and anus. UC may occur in people of any age, but most often it starts between the ages of 15 and 30 years, or less frequently between 50 and 70. Children and adolescents sometimes develop the disease. UC affects men and women equally and appears to occur in some families. Clinical and epidemiological data do not support a simple Mendelian model of inheritance for IBD. CD and UC are considered to be complex

polygenic diseases. UC is a chronic disease in which the large intestine becomes inflamed and ulcerated (pitted or eroded), leading to flare-ups (bouts or attacks) of bloody diarrhea, abdominal cramps, and fever. The long-term risk of colon cancer is increased[1-3].

To make a diagnosis of IBD, physicians must first exclude other possible causes of inflammation. For example, infection with parasites or bacteria may also cause inflammation. Therefore, several tests should be performed. Stool samples are analyzed for evidence of a bacterial or parasitic infection (e.g. acquired during travel), including a type of bacterial infection (*Clostridium difficile* infection) that can result from antibiotic use, and sexually transmitted diseases of the rectum, such as gonorrhea, herpes virus infection, and chlamydia infection. Tissue samples (biopsies) may be taken from the lining of the rectum during sigmoidoscopy (an examination of the sigmoid colon using a viewing tube) and examined microscopically for evidence of other causes of colon inflammation (colitis). Other possible causes of similar abdominal symptoms that should be excluded are ischemic colitis, which occurs more often in people older than 50 years; certain gynecological disorders; celiac disease; and irritable bowel syndrome[1].

In traditional Chinese medicine (TCM), UC is a systemic disease that affects many parts of the body, although patients mainly manifest with intestinal symptoms. In TCM principles, the problem is closely associated with organ dysfunction, in particular the *pi* (spleen), that causes a failure to self-regulate the intestinal environment. TCM specialists generally agree that constitutional weakness, invasion of exogenous pathogens, an unbalanced diet and emotional factors all contribute to the development of the problem.

Genetic changes of patients with UC have been demonstrated. What are the changes in Chinese UC patients? There are gene diversification analyses of Chinese patients with UC in the recent literature.

ETIOLOGY AND EPIDEMIOLOGY

Etiology

The etiology of UC is unknown. The consensus is, so far, that it is a response to environmental triggers (infection, drugs or other agents) in genetically susceptible individuals. The genetic component is not as strong in UC as in CD. However, 10%-20% of patients with UC have at least one family member with IBD[1-2]. There are marked differences between ethnic groups and some (such as Ashkenazi Jews) have a particularly high incidence. Non-steroidal anti-inflammatory drugs may cause an episode of acute active disease in some patients with IBD. UC primarily affects young adults, but it can occur at any age from 5 to 80 years and women tend to be more commonly affected than men. It is a worldwide disorder, and the high-incidence areas include the United Kingdom, the United States, northern Europe and Australia. Low-incidence areas include Asia, Japan, and South America. The causes of UC remain unknown. The major theories are associated with infection, allergy to food component, genetics, environmental factors, and immune response to bacteria or other antigens[1].

Typical symptoms during flare-ups include abdominal cramps, an urge to move the bowels, and diarrhea (typically bloody). The diagnosis is based on an examination of the sigmoid colon using a flexible viewing tube (sigmoidoscopy) or an examination of the large intestine using a flexible viewing tube (colonoscopy). People who have had UC for a long time may develop colon cancer. Treatment is aimed at controlling the inflammation, reducing symptoms, and replacing any lost fluids and nutrients. UC may start at any age but usually begins between the ages of 15 and 30 years. A small group of people have their first attack between the ages of 50 and 70 years. UC usually does not affect the full thickness of the wall of the large intestine and hardly ever affects the small intestine. The disease usually begins in the rectum or the rectum and the sigmoid colon (the lower end of the large intestine) but may eventually spread along part or all of the large intestine. UC, which is confined to the rectum, is a very common and relatively benign form. In some people, most of the large intestine is affected early.

Epidemiology

UC affects about one in 1000 people in the Western world. Peak incidence is between the ages of 10 and 40 years. UC may affect people of any age and 15% of people are over the age of 60 years at diagnosis[1-4]. The incidence of UC in North America is 10-12 cases per 100 000, with a peak incidence of UC occurring between the ages of 15 and 25 years. There is thought to be a bimodal distribution in age of onset, with a second peak in incidence occurring in the sixth decade of life. The disease affects females more than males, with highest incidences in the United States, Canada, the United Kingdom, and Scandinavia. Higher incidences are seen in the northern parts compared to the south in Europe and the United States.

Rising incidence and prevalence of UC have been observed in Asian countries. Lok *et al*[2] conducted a study in an Asian center, aiming to describe the epidemiology and clinical characteristics of UC in the local Chinese population. This is a retrospective analysis of patients with diagnosis of UC in our hospital from June 1990 to December 2006. The diagnosis of UC has to satisfy the internationally accepted criteria. All patients were Chinese residents in a well-defined catchments' area. Clinical and epidemiological data were obtained from medical records and patient interviews. Seventy-three Chinese UC patients were managed in hospital. The hospital-based prevalence has risen by three times over a 10-year period, but no definite rising incidence can be demonstrated. The mean age at diagnosis was 40.6 years and the median duration of disease was 72 mo. In the patient cohort, 38.4% had ulcerative proctitis and 26% had left-sided UC, whereas 35.6% had extensive UC at presentation. The majority presented with mild (39.7%) or moderate (30.2%) disease activity, but 27.4% presented with severe disease. One of the two patients (2.7%) with fulminant disease developed

toxic megacolon. Extra-gastrointestinal manifestations occurred in 13.7%. During the follow-up period, most patients (86.3%) were in disease remission. Four patients (5.5%) underwent colectomy, four (5.5%) died, and two (2.7%) were lost to follow-up. The prevalence but not the incidence of UC is rising in the Chinese population. It usually affects young patients and a substantial proportion of patients presented with severe and fulminant disease. The disease activity of most Chinese patients can be controlled with medical treatment, though a small proportion of patients need surgery or have a fatal outcome[2-4].

Jin *et al*[3] explored the indications for colonoscopic examination and the distribution of diagnostic diseases. From January 2000 to December 2004, 5960 patients received colonoscopic examination in a colorectal center. The indications for colonoscopic examination and the distribution of its diagnostic diseases were analyzed. There were 3096 males and 2594 females, and the mean age was 52 ± 15 years. The reasons for colonoscopy included hemafecia (26.9%), atypical abdominal pain (25.8%), diarrhea or increased frequency of stools (11.1%), anal tenesmus or discomfort (7.6%), constipation (7.0%), mucous or bloody purulent stools (3.0%), intra-rectal mass or abdominal mass on physical examination (0.9%), re-examination after colonoscopic polypectomy (10.9%), re-examination after operation for colorectal cancer (1.5%), and simple health examination (2.2%). Colonoscopy reached the cecum in 97.7% of the cases, and at least one disease was found in 2283 cases (40.1%). Among them, colorectal cancer accounted for 10.3%, colorectal polyps 19.6%, UC 4.3%, and CD 0.5%. The indications for colonoscopy are too strict to screen for early stage colorectal cancer. Colonoscopy should be performed in patients with symptoms such as bloody stools, diarrhea, abdominal pain, constipation, or with colorectal polyps, after operation for colorectal cancer, or as members of a hereditary colorectal cancer family[3]. Cigarette smoking, alcohol use, appendectomy, and family history of IBD have all been shown to be associated with IBD, but there was no report of risk factors for IBD in a Chinese population, in which the incidence of IBD has increased during the past decade. Jiang *et al*[4] conducted a case-control study to examine associations between previously reported environmental risk factors and development of UC in Wuhan city, central China. A total of 177 patients with UC and 177 age-matched and sex-matched controls were prospectively studied in Wuhan city from January 2004 to December 2004. An age-matched and sex-matched case-control study was conducted to assess the role of smoking, alcohol use, appendectomy, and other potential risk factors in the development of UC, by a detailed questionnaire. Smoking was a protective factor and ex-smoking is a risk factor for UC (compared with non-smokers, smokers: $P = 0.0001$; ex-smokers: $P = 0.008$). Positive family history of IBD was a risk factor ($P = 0.025$), whereas appendectomy was a protective factor ($P = 0.028$) for UC. There were no significant associations between UC and other factors examined. Although the incidence of UC in Chinese is relatively lower than that in whites, the same risk factors for UC that were reported in white populations were associated with Chinese UC patients. Specifically, smoking was a protective factor for UC and ex-smoking was associated with an increase risk of UC in a Chinese population. Family history of IBD was shown to be a risk for UC, whereas appendectomy was associated with a low risk for UC[2,4].

Investigative papers about IBD in Chinese medical journals from 1989 to 2003 were reviewed to understand the progress of basic IBD research in China, by Bai *et al*[5]. The basic science investigative papers about IBD from 1989 to 2003 in Chinese periodicals (VIP and CMCC) were reviewed and analyzed; the key words used were as follows: IBD, UC, CD, basic science investigation, and literature review. There were 3454 articles about IBD published in Chinese medical journals from 1989 to 2003, and during these 15 years, 508 papers focused on basic scientific investigations. There were 463 papers investigating the pathogenesis of IBD, 287 papers on immunological mechanisms, and 176 papers about other mechanisms. There were 142 papers investigating the mechanisms of TCM in IBD from 1989 to 2003, which included 117 papers related to animal experiments and 25 papers related to clinical studies. There have been relatively few investigative scientific papers on IBD published in Chinese medical journals. However, the study of IBD has been emphasized in China. Research on the immunological mechanisms of IBD has been predominant. Furthermore, a large number of the research papers are about the mechanisms and effects of TCM in IBD.

IBD had been uncommon in China until about 1990, but since then, more and more cases have been seen in clinical settings. The prevalence and phenotype of IBD in the Chinese population is not well known. One study investigated the trend in prevalence of UC and CD in Wuhan city, central China, and evaluated the clinical features, extraintestinal manifestations, and the treatment of IBD in the last 14 years. Three hundred and eighty-nine patients with UC and 63 with CD were retrospectively collected from five central hospitals in Wuhan city, in which high-quality endoscopic and histological diagnoses were available from 1990 to 2003. UC and CD were diagnosed based on clinical, laboratory, radiological, endoscopic and histological examinations according to the internationally accepted Lennard-Jones criteria. The trend toward prevalence of UC and CD increased between 1990 and 2003 in Wuhan city. There was no change in the sex and age distribution comparing the two periods of 1990-1996 and 1997-2003, both in UC and CD. However, the number of individuals with higher education and a professional occupation from 1997 to 2003 was significantly higher than that during the period from 1990 to 1996 in patients with UC ($P \leqslant 0.004$). The mean age of patients with CD was significantly younger than that of UC at the time of diagnosis ($P < 0.0001$). The ratio of male to female patients was 1.53:1 in UC and 2.32:1 in CD, respectively.

The mean duration of onset of the disease to diagnosis was 1.4 years in UC and 1.1 years in CD. The extra-intestinal manifestations of UC and CD were 5.7% and 19%, and complications of UC and CD were 6.4% and 50.8%, respectively. Only 3% of UC patients required surgery, whereas 27% of CD patients underwent surgical procedures ($P < 0.001$). The prevalence of IBD has increased in Wuhan city, central China, but is not as high as in Western countries. The disease in Wuhan city has often been associated with young adult professional males with a high level of education. The clinical presentation of UC was often mild and had few extra-intestinal manifestations[4-6].

GENE MARKERS OF ULCERATIVE COLITIS IN THE CHINESE

Genetic factors play a significant role in determining IBD susceptibility. Many genes play a vital role in the development of IBD, including *TNF-308A*, *CARD15 (NOD2)*, *MIF-173*, *N-acetyltransferase 2 (NAT2)*, *NKG2D (natural killer cell 2D)*, *STAT6 (signal transducer and activator of transcription 6)*, *CTLA-4 (cytotoxic T lymphocyte antigen-4)*, *MICA-MICB (major histocompatibility complex A and B)*, *HLA-DRB1*, *HLA class-Ⅱ*, *IL-18* (*interleukin-18*), *IL-4*, *MICA-A5*, *CD14*, *TLR4*, *Fas-670*, *p53* and *NF-κB*.

TNF-308A

Tumor necrosis factor α (TNFα) is a pro-inflammatory cytokine that plays an important role in mediating inflammation and has been implicated in the pathogenesis of IBD. The regulation of TNF expression is genetically determined. The TNF gene lies on the short arm of chromosome 6 (6p21), 250 kb from the center of human leucocyte antigen-B (HLA-B), between HLA-B and DR, and within HLA Ⅱ. Recent studies have evaluated the role of TNF promoter polymorphisms in IBD but data are inconsistent. To date, few studies have reported the association of TNF promoter polymorphisms with susceptibility to UC in the Chinese Han ethnic population. Trans-racial mapping in an ethnically distinct but homogenous population may help clarify these associations. There is an association between TNF promoter polymorphisms and the susceptibility to UC in the Chinese Han ethnic population by genotyping for 6 common TNF promoter polymorphisms[6-9].

In a large sample study, a strong association between UC patients and the TNF-308A polymorphism was found in Japanese subjects[7]. No conclusive data on this association in European patients exist, however. This may reflect that the associations of TNF promoter polymorphisms with susceptibility to UC do vary among ethnic groups. Some scientists have supposed that TNF polymorphism is increased in IBD patients, even more than NOD2, and plays a more important role in the Asian population. IBD in the Asian population has unique epidemiological and clinical characteristics. For example, UC has a higher morbidity than CD in Asia. In an eastern China hospital between 1994 and 2003, 379 patients were diagnosed to have IBD. Of 379 patients, 317 had UC (83.6%) and this study shows similar characteristics of IBD to that in the West. However, there are some differences with respect to low severity and less extra-intestinal manifestations[8]. The ethnic and geographic differences may give important clues to the etiology of IBD. In the final analysis, genetic background plays a key role. To date, few studies have reported the association of TNF promoter polymorphisms with susceptibility to UC in the Chinese Han ethnic population. A number of studies have reported a high population attributed risk percentage of TNF promoter single nucleotide polymorphisms (SNPs), which reflects the higher mutant-type frequency in UC.

The importance of TNFα and the TNF receptor gene polymorphisms in the etiopathogenesis of IBD has not been elucidated. DNA from peripheral blood samples was obtained from 124 patients with CD, 106 patients with UC, and 111 unrelated healthy controls. Two SNPs of the *TNFα* gene, TNF (-308 G/A and -238 G/A), an SNP of the TNF receptor superfamily member 1A gene, TNFRSF1A (also known as TNFR1), at codon 12 in exon 1 (CCA/CCG), and two SNPs of the 1B gene, TNFRSF1B (also known as TNFR2), (1466 A/G and 1493 C/T) were examined. There was a difference in the carrier frequency for haplotype AG (-308 A, -238 G) between UC patients and the controls ($P < 0.01$). There was also a significant difference in carrier frequency for haplotype AT (1466 A, 1493 T) of the *TNFRSF1B* gene between CD patients and the controls ($P < 0.01$), and in those who were poor responders to treatment, which consisted of nutritional therapy, medical therapy and surgical therapy ($P < 0.001$). The authors suggest that one of the genes responsible for UC may be the *TNF* gene, or an adjacent gene, and that *TNFRSF1B* gene polymorphisms contribute greatly to the increased onset risk of CD and to the disease behavior[7].

Numerous studies from Europe and North America have provided a wealth of information regarding the epidemiological and clinical characteristics of IBD in Caucasians. Previous studies in mainland China have been limited by small patient numbers or by lack of detailed information about clinical subgroups of the disease. Cao *et al*[8] have assessed the demographic and clinical characteristics of IBD in Chinese patients. In the Sir Run Shaw Hospital between 1994 and 2003, 379 patients were diagnosed as having IBD. Demographic and clinical data were collected and analyzed. Of 379 patients, 317 had UC (83.6%, 168 male, 149 female, a male:female ratio of 1.13:1, age range at diagnosis 14-79 years, mean age 44 years) and 62 had CD (16.4%, 39 male and 23 female, a male:female ratio of 1.70:1, age range at diagnosis 13-70 years, mean age 33 years). In UC, 11.4% of patients had proctitis, 25.2% had proctosigmoiditis, 18.6% had disease in the splenic flexure and 44.8% had extensive colitis. Nine patients with UC (2.8%) had arthritis, and three (0.9%) had iritis or conjunctivitis. Of the 62 CD patients, 16 (25.8%) had diseases restricted to the terminal ileum, 15 (24.2%) had colonic diseases,

20 (32.3%) had ileocolonic disease and 11 (17.7%) had disease involving the upper gastrointestinal tract. This study showed similar characteristics of IBD to that in the West, but there are some differences with respect to severity and extra-intestinal manifestations. The ethnic and geographic differences may give important clues to the etiology of IBD[8].

Cao *et al*[9] reported the association with TNF-308A polymorphisms in Chinese patients with UC, suggesting that *TNF* gene may be a susceptibility gene for UC. The production of TNFα is elevated in TNF-308A carriers, resulting in excessive inflammation and onset of UC. The clinical application is to apply this new genetic information in the clinical setting to allow more rational therapies, selecting effective therapies (e.g. anti-TNF antibody) for refractory patients with UC, based on the genetic background. Further studies will be required to determine the functional effects of TNF-308A. Hereafter, authors can study gene knock-out mice, estimate the expression of TNFα in mutant cells using Northern and Western blotting, and investigate TNFα secretion by mutant-type cells after stimulation with LPS, compared with wide-type. We can also carry out a cohort study on correlation of TNFα expression level with *TNF-308A* genes and efficacy of anti-TNF antibody in UC patients based on the genetic background, in order to ascertain whether TNF-308A is responsible for UC[9].

Recent studies have evaluated the role of TNF promoter polymorphisms in IBD, but the data are inconsistent. Trans-racial mapping in an ethnically distinct but homogenous population may help clarify these associations. Cao *et al*[9] investigated the association between TNF promoter polymorphisms and susceptibility to UC in the Chinese Han ethnic population. They studied 110 unrelated UC patients and 292 healthy controls from Zhejiang Province, China. Genotyping for six common TNF promoter polymorphisms (TNF -1031T/C, -863C/A, -857C/T, -380G/A, -308G/A, -238G/A) was carried out by polymerase chain reaction sequence-specific primers (PCR-SSP). TNF -857T was increased in patients but without statistical significance ($P = 0.06$). Haplotype analysis revealed six haplotypes including two (H5 and H3), which contained TNF-308A. Of note, the rare haplotype H3 has not previously been identified in Caucasian populations. Homozygosis for the haplotype H4 comprising the common alleles at each TNF promoter single-nucleotide polymorphism (SNP) was negatively associated with disease ($P < 0.05$). The association with TNF-308A polymorphisms in Chinese patients with UC was reported by Cao *et al*[9]. The functional study in Chinese Han ethnic population is now required.

Progressive venous stenosis mediated, in part, by inflammatory cytokines is a major cause of synthetic hemodialysis graft failure. A TNFα gene polymorphism (G to A, position -308) has been shown to increase plasma cytokine levels and severity of diseases with an underlying inflammatory component. The TNFα-308 G/A and the TNFβ NcO1 polymorphisms have both been described to be associated with survival in sepsis or septic shock of various origins. That the TNFB2/TNFB2 genotype of the TNF-β NcO1 polymorphism is significantly associated with an increased risk for development of severe sepsis in severely injured blunt trauma patients was recently reported[9]. Up to now, neither functional consequences associated with these polymorphisms in inflammation nor the relationship of the TNFα -308 G/A polymorphism to the development of severe sepsis and its significance compared with the TNFβ NcO1 polymorphism have been determined for trauma patients.

The TNFα-308 allele may be related to susceptibility to UC. The *TNFα-308* gene polymorphism is not involved in pathogenesis of CD. No correlation was found between the TNFβ+252 polymorphism and IBD. Polymorphisms of the TNFα-308 and TNFβ+252 loci do not correlate with age, gender, disease activity or lesion site[8-10]. PCR and restriction fragment length polymorphism (RFLP) techniques were used to analyze gene polymorphisms in the *TNFα* and *TNFβ* genes in 131 patients with IBD[10]. The genotype frequency and allelic frequency of TNFα-308 in patients with UC were 15.5% and 8.7%, respectively, significantly higher than the control population ($P < 0.001$). There was no significant difference between patients with CD and the normal population with regard to the genotype frequency and allelic frequency of TNFα-308, and neither were there any differences with regard to TNFβ+252 in patients with IBD (UC and CD) and the normal population. The TNFα-308 polymorphism and the TNFβ+252 loci did not correlate with age, gender, disease activity or lesion site for IBD patients.

CARD15 (NOD2)

A lot of research has been undertaken on the genetic susceptibility of IBD. Genome-wide linkage studies focused on more than 10 chromosomal regions and fine-mapping of these regions have identified a number of genes, including *CARD15 (NOD2)*, *DLG5*, *OCTN1* and *2*, *TLR4* and *CARD4 (NOD1)*. With the recent completion of the human genome project, whole genome association studies (WGAS) have become possible and additional genes (*IL23R, IRGM, PTGER4, ATG16L1*) for CD and UC have been identified. At present, the *CARD15* gene is still the best understood susceptibility gene, explaining around 20% of the genetic predisposition to CD. Prediction of disease phenotype and response to the main therapies has for many years been a goal for physicians treating IBD patients. We now can accumulate some evidence, proving that genetic factors indeed influence both the clinical course of IBD patients and their likelihood of responding to certain therapies. Henckaerts *et al*[11] expected an exponential increase in the efforts devoted to research in this area. The optimal prediction of both disease behavior and response to therapy might result from combinations of clinical, biochemical, serological and genetic factors.

An insertion mutation at nucleotide 3020 (3020insC) in the caspase recruitment domain gene (*CARD15*),

originally reported as NOD2, is strongly associated with CD. The C-insertion mutation at nucleotide 3020 (3020inC) in the leucine-rich repeat (LRR) region results in a frameshift in the tenth LRR followed by a premature stop codon. This truncation mutation is responsible for the inability to activate nuclear factor (NF)-κB in response to bacterial lipopolysaccharide (LPS). The authors aimed to genotype *NOD2/CARD15* gene 3020insC frameshift mutation in Chinese patients with IBD. Guo *et al*[12] genotyped an insertion polymorphism affecting the leucine-rich region of the protein product by the allele-specific PCR in 74 unrelated patients with UC of Han nationality in Hubei Province of China, 15 patients with CD, and 172 healthy individuals. No significant differences were found in the genotype and allele frequencies of the C-insertion mutation of *NOD2* gene among patients with CD and UC and healthy controls. *NOD2* gene 3020insC frame-shift mutation is not a major contributor to the susceptibility to both UC and CD in Chinese Han patients[12].

The SNPs distribution of NOD2/CARD15 (R702W, G908R), OCTN1 1672C/T and OCTN2-207G/C in Chinese patients with IBD was investigated[13]. A total of 151 patients with UC, 61 patients with CD and 200 unrelated healthy controls were genotyped. Genotyping was performed by PCR-SSP or by RFLP analysis. Among the subjects in their study groups, including patients with CD, UC and healthy controls, none had OCTN and CARD15 variants, and very rarely, an IBD family history was found in their patients with the percentage of 0 (0/61 CD) and 1.3% (2/151 UC). The results indicate that although OCTN or CARD15 variation is associated with susceptibility to IBD in Western populations, these might be rare and may not be associated with susceptibility to IBD in Chinese patients[13].

The common variants in NOD2/CARD15 found in Caucasians with CD are not associated with CD in the Chinese Han population[14]. The three previously described SNPs associated with the development of CD in Caucasians are not found in Chinese patients with CD. None of the patients with CD had heterozygous or homozygous SNP variants. Similarly, none of the UC or dyspeptic controls had these SNPs[15].

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 CD and several *HLA* genes are associated with UC and CD. Toll like receptors (TLRs) play an important role in the innate immune response against infections by mediating recognition of pathogen-associated microbial patterns. Studying 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, such as NOD1/caspase-activation recruitment domains 4 (CARD4), chemokine ligand 20 (CCL20), IL-11, and IL-18. The characterization of these novel genes will lead to the identification of therapeutic agents and clinical assessment of phenotype and prognosis in patients with IBD[16].

MIF-173 gene

The etiology and pathogenesis of IBD is still unclear, but it has become evident that immune and genetic factors are involved in the process of IBD. Some cytokine gene polymorphisms such as TNFα, IL-1β and IL-1RA are known to be commonly associated with IBD. Macrophage migration inhibitory factor (MIF) is an important pro-inflammatory cytokine and plays a critical role in immune and inflammatory responses. MIF is implicated in a large number of immune and inflammatory diseases, including asthma, chronic hepatitis B, allergic neuritis and rheumatoid arthritis. Plasma MIF was reported elevated in patients with UC or CD compared with healthy controls. Anti-MIF antibodies reduced the severity of experimental colitis and limited the up-regulation of Th1-type cytokines. Anti-MIF antibodies are therefore of a potential therapeutic use in IBD. In the T lymphoblast cell line, the reverse situation was found with the MIF-173*C, significantly increasing the MIF expression under basal conditions. These differences in expression are likely to be due to differences in transcription factor interaction with the MIF-173 element. AP-4 transcription factor is a particular candidate[1,17] based on the promoter sequence analysis.

MIF-173 SNP was genotyped by tetra-primer amplification refractory mutation system (ARMS) and RFLP-PCR was also performed in 142 healthy subjects and 98 patients with IBD[17]. There were no discrepancies between the results obtained by tetra-primer ARMS and RFLP-PCR. The frequency of MIF-173 CC genotype was significantly higher in patients with UC (15.5%) than in healthy individuals (5.6%, $P = 0.018$). There was a trend towards a higher frequency of CC genotype among CD patients compared with healthy controls, however, this did not attain statistical significance ($P = 0.245$). MIF-173 CC genotype may be associated with susceptibility to UC. The results showed that the frequency of MIF-173 CC genotype was significantly higher in patients with UC than in healthy individuals. It suggested that MIF-173 CC genotype could be associated with susceptibility to UC. However CC genotype was not related to clinical features in patients with UC in Chinese Han population[17].

NAT2

Polymorphisms of NAT2 (N-acetyltransferase 2) acetylation may influence drug toxicity and efficacy and are associated with differential susceptibility to cancer. Acetylation phenotype may have clinical implications. NAT 2 is an enzyme that catalyzes the acetylation of harmful arylamines and has been implicated in various types of cancer. NAT 2 is primarily expressed in the hepatic system and intestinal epithelium, and is encoded at two polymorphic loci that give the phenotypic characteristics of slow and fast acetylation[18].

Arylamine NATs are xenobiotic-metabolizing enzymes responsible for the acetylation of many aromatic arylamine and heterocyclic amines, thereby playing an important role in both detoxification and activation of numerous drugs and carcinogens. Two closely related isoforms (NAT1 and NAT2) have been described in humans. NAT2 is mainly expressed in liver and gut, whereas NAT1 is found in a wide range of tissues. Inter-individual variations in NAT genes have been shown to be a potential source of pharmacological and/or pathological susceptibility. In addition, there is evidence that non-genetic factors, such as substrate-dependent inhibition, drug interactions or cellular redox conditions may also contribute to NAT activity. The recent findings provided possible mechanisms by which these environmental determinants may affect NAT activity. Interestingly, these data could contribute to the development of selective NAT inhibitors for the treatment of cancer and microbial diseases[18].

The wild type allele (NAT2*4) and three variant alleles (NAT2*5B, NAT2*6A and NAT*7B) of the *NAT2* gene were determined in 101 patients with IBD (84 patients with UC and 17 patients with CD) and 109 healthy controls by the RFLP-PCR method. Sixty-eight patients with IBD treated with SASP were followed up, and their adverse reactions were recorded. Eleven patients (16%) experienced adverse effects from SASP, including nine cases of sulfapyridine (SP) dose-related adverse effects and two cases of hypersensitivity (skin rash). Patients with the slow acetylator genotypes without the NAT2*4 allele experienced adverse effects more frequently (36%) than those with the fast acetylator genotypes with at least one NAT2*4 allele (11%), but the results were not significantly different ($P = 0.051$). However, those with the slow acetylator genotypes experienced more SP dose-related adverse effects than those with the fast acetylator genotypes ($P = 0.019$). The NAT2 gene polymorphism was not associated with susceptibility to IBD in Chinese populations, but the NAT2 slow acetylator genotypes were significantly associated with SP dose-related adverse effects of SASP in the treatment of IBD[19].

NKG2D

NKG2D (natural killer cell 2D) is an important activated cytokine that has been implicated in inflammatory reactions and the immune response. One of the best characterized NK cell receptors is NKG2D, a highly conserved C-type, lectin-like membrane glycoprotein expressed on essentially all NK cells, as well as on γδ-TcR+ T cells and αβ-TcR+ CD8+ T cells, in humans and mice. Recent studies implicating NKG2D in T cell and NK cell-mediated immunity to viruses and tumors, and its potential role in autoimmune diseases and allergenic bone marrow transplantation have been reviewed. NKG2D is a major activation receptor that associates with novel activation motif with DAP10 or ITAM containing KARAP/DAP12 adaptor molecules. A fundamental question is whether NK cell activation initiated *via* the H60-NKG2D interaction overrides the negative inhibition generated by the engagement of MHC class I to Ly49 receptors. Although an altered balance in the signaling strength of activating and inhibiting pathways of NK cells has been previously postulated. Recent findings illustrate that NK cell activation *via* NKG2D receptor can occur despite the normal expression of MHC class I molecules on the target cells[10-12]. By varying the levels of H60 expression and introducing additional MHC class I molecules on the target cells, Cao *et al*[20] demonstrated that the inhibitory Ly49 receptors can down-regulate NKG2D-mediated NK cell functions.

The function and the location of NKG2D gene show that it is an ideal susceptibility gene for UC. Cao *et al*[20] evaluated the NKG2D gene polymorphisms in patients from Zhejiang Province to determine whether the gene is associated with susceptibility to UC in the Chinese Han population. Blood samples were obtained from 110 patients with UC and 292 healthy controls in Zhejiang. Genotyping for two common NKG2D (10676G/, 908A/) polymorphisms was carried out using polymerase chain sequences with specific primers. NKG2D was not associated with disease (908A allele frequency 19.1% in patients *vs* 16.3% in controls, $P > 0.05$). Neither the patients with UC nor healthy controls had 10676G heterozygous or homozygous variants. The common variants in NKG2D are not associated with UC in the Chinese Han population. Research of larger samples and analysis from different layers and DNA sequences will help determine the function of NKG2D in the process of UC[20].

STAT6

STAT6 (signal transducer and activator of transcription 6) is a human gene. The protein encoded by this gene is a member of the STAT family of transcription factors. In response to cytokines and growth factors, STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. This protein plays a central role in exerting IL-4-mediated biological responses. IL-4 and IL-13 share many biological activities. To some extent, this is because they both signal *via* a shared receptor, IL-4Rα. Ligation of IL-4Rα results in activation of STAT6 and insulin receptor substrate (IRS) molecules. In T and B cells, IL-4Rα signaling contributes to cell-mediated and humoral aspects of allergic inflammation. It has recently become clear that IL-4 and IL-13 produced in inflamed tissues activate signaling in normally resident cells of the airway.

The *STAT6* gene is located on chromosome 12q13.3-14.1, just within the IBD2 region and is a key transcription factor involved in IL-4- and IL-13-mediated Th2 response. The G2964A polymorphism in the 3' untranslated region of the *STAT6* gene was studied in 84 unrelated Chinese patients with UC and 176 healthy controls by PCR and the amplification created restriction site method. The results were then compared with those from a Dutch study published previously. Significant

differences in genotype and allele frequencies of the STAT6 G2964A polymorphism were not found between patients with UC and healthy controls. Subgroups of the patients with UC classified according to the age at onset, sex and location of disease did not differ significantly in the distribution of this polymorphism. However, the genotypes ($P < 0.0001$) and allele frequencies ($P < 0.0001$) were significantly different between the Chinese and Dutch populations. The STAT6 G2964A polymorphism is not involved in the genetic susceptibility of Chinese patients to UC[21].

CTLA-4

CTLA-4 (cytotoxic T-lymphocyte antigen 4) is a glycoprotein expressed in activated T cells. CTLA-4 is essentially a costimulatory receptor that controls activation of T cells. In contrast to CD28, CTLA-4 delivers negative signals to T cells. *CTLA-4* gene is located on chromosome 2q33 and three *CTLA-4* gene polymorphisms in exon 1 (adenine or guanine at position) and in promoter -318, and a microsatellite (AT)n marker at position 642 of the 3'-untranslated region of exon 3. Recently, several independent studies reported a reduced inhibitory function of CTLA-4 in individuals with certain CTLA-4 genotypes. CTLA-4 consists of four exons that encode leader peptide, ligand-binding domain, transmembrane domain, and cytoplasmic tail, respectively. In humans, there are two isoforms of CTLA-4, which are a full-length isoform (flCTLA-4 transcript) and a soluble isoform (sCTLA-4 transcript) which lacks exon 3 by alternative splicing. Especially, sCTLA-4 is secreted and circulating in human sera. CTLA-4 is a negative regulator of T-cell proliferation and activation, which plays a critical role in the induction of self-tolerance and mediates antigen-specific apoptosis. Type 1 diabetes is a T-cell mediated autoimmune disease, therefore, its onset is partly associated with deficient expression and function of CTLA-4. Recent findings suggest that programmed cell death may also be involved in the pathogenesis of type 2 diabetes. Furthermore, there is evidence favoring a convergence in signaling pathways toward common effectors of beta-cell apoptosis elicited by stimuli implicated in the pathogenesis of type 1 and type 2 diabetes. If CTLA-4 were involved in this process, its association with type 2 diabetes might be conceivable. A functional role of the CTLA-4 A/G polymorphism, encoding a threonine to alanine change within the signal peptide of CTLA-4, has several possible explanations. It may be in linkage disequilibrium with the (AT)n microsatellite in the 3'-untranslated region and could, therefore, affect RNA stability. Equally, it may be in linkage disequilibrium with other disease-causing mutations[22-23]. However, it is also possible that this signal peptide polymorphism determines a subtle alteration in the subcellular localization of mature CTL A-4 protein, or affects the interaction of the nascent peptide with chaperonins, leading to a functionally important difference in the folding of the mature protein.

Eighty-seven patients with UC and 116 healthy controls were genotyped for CTLA-4 promoter -1722 and -1661 polymorphisms by RFLP-PCR in Hubei Province of central China. The frequency of "A/G + G/G" genotype at the -1661 site was significantly higher in UC patients than in healthy controls ($P = 0.002$). The frequency of the G allele at the -1661 site was also significantly higher in UC patients than in the controls ($P = 0.002$). However, the distribution of the genotypes at the -1722 site was not significantly different between the UC patients and the controls. The G allele of CTLA-4 promoter -1661 polymorphism showed a highly significant association with UC in the Han Chinese of central China[22]. One hundred unrelated Chinese patients with UC and 140 healthy controls were studied. The (AT) repeats in the 3' untranslated region of exon 4 of the CTLA-4 gene were amplified by allele-specific PCR. The amplified products were electrophoresed on a 120 g/L polyacrylamide gel, followed by silver staining. Twenty alleles were found in Chinese patients and healthy controls. The 122-bp allele was increased in UC compared with healthy controls ($P = 0.0001$). The frequency of the longer alleles ($\geqslant$ 118 bp) of UC was higher than that in healthy controls ($P = 0.0001$), but was not associated with location and severity of the disease. Furthermore, the longer alleles were not associated with haplotypes of C-318T/A+49G of the *CTLA-4* gene in Chinese patients with UC. The longer alleles of the *CTLA-4* gene microsatellite polymorphism were strongly associated with UC in Chinese patients[23].

Hou *et al*[24] studied 82 unrelated patients with UC and 204 healthy controls in a Chinese Han population. The frequency of the haplotype 2, 3 (-318C+49G/-318T+49A) was 26% in patients with UC and 41% in healthy controls ($P = 0.0147$), but this significance disappeared when Bonferoni correction was applied. No other significant differences in the distribution of allele and genotype frequencies were observed between *C-318T* and *A+49G* gene polymorphisms in UC of the Chinese Han population. The C-318T and A+49G polymorphisms of the *CTLA-4* gene were not associated with UC in Chinese Han patients[24].

CTLA-4 expressed mainly on activated T cells, inhibits T cell activation by combining B7 through competing CD28 and maintains immune system homeostasis. Polymorphisms in the *CTLA-4* gene are known to be associated with several autoimmune diseases, but no studies have related them to IBD. Sixty-eight unrelated Chinese Han patients with IBD (54 UC and 14 CD) and 140 healthy controls were studied. The (AT)n repeat sequence in the 3' untranslated region of exon 4 was amplified by allele-specific PCR. The amplified products were electrophoresed by 120 g/L polyacrylamide gel, followed by silver staining. Eighteen alleles of CTLA-4 microsatellite were found in Chinese patients and healthy individuals. A long allele of 122 bp was apparently increased in patients with UC compared with healthy controls ($P = 0.0002$). *CTLA-4* gene microsatellite polymorphism was strongly associated with UC in Chinese Han patients in Hubei Province[25].

Xia *et al*[26] studied 139 unrelated patients with UC, 163 patients with CD and 174 healthy controls of Dutch

Caucasian origin, as well as 35 patients with UC and 62 healthy controls from the Chinese Han population. No significant differences in the distribution of allele, genotype and haplotype frequencies were observed between *C-318T* and *A+49G* gene polymorphisms and IBD in Dutch Caucasians and UC in the Chinese Han population. Although the haplotypes of the C-318T and A+49G polymorphisms were distributed differently between Dutch Caucasian and Chinese Han populations, there were no differences in the subgroups of patients with CD classified according to age, localization and behavior in the Vienna classification and in those with UC classified according to age at onset, disease extension and presence of colectomy in the Dutch patients. However, the CTLA4-318 genotype CC was more frequent in patients with CD over 40 years of age (93%) than in younger patients (74%, $P = 0.045$). *C-318T* and *A+49G CTLA4* gene polymorphisms and their haplotypes are not associated with IBD in Dutch Caucasian patients and with UC in Chinese patients[26]. CTLA-4 polymorphism is not associated with UC in the Iranian population[27].

MICA-MICB

The 6D4 monoclonal antibody reacts with the human major histocompatibility complex (MHC) class I -related molecules A and B (MICA and MICB). MICA and MICB are related proteins of 83% amino acid similarity, and show homology with classical human leukocyte antigen (HLA) molecules. The structure of MICA and MICB is similar to classical HLA class I chains, however they do not bind β2 microglobulin or peptide typical of HLA class I. MICA and MICB are expressed on the cell surface of endothelial cells, fibroblasts, gastric epithelium and PHA-stimulated T cells, and act as a ligand for NKGD2 expressed on the surface of NK cells, γδ T cells and αβ CD8+ T cells. There is evidence suggesting that human cytomegalovirus subverts NK cell detection by inhibiting the function of MICB. Furthermore, MICA and MICB expression has been detected in several epithelial tumors isolated from breast, lung, ovary, prostate, colon and kidney. The MIC genes, which were described independently by two groups of investigators in 1994, encode proteins that are remotely similar to the HLA class I gene products. However, the MIC proteins do not associate with β2-microglobulin and have a groove that is too narrow to accommodate peptides for presentation to T cells.

MICA and MICB are stress-inducible cell surface antigens recognized by immunocytes bearing the receptor NKG2D, including intestinal epithelial Vδ1 γ/δ T cells, which may play a role in immunological reaction in intestinal mucosa. Lu *et al*[28] investigated the association of the microsatellite polymorphisms in the intron 1 of MICB and the MICA-MICB haplotype with the susceptibility to UC in the Chinese population. The microsatellite polymorphisms of MICB were genotyped in unrelated 127 Chinese patients with UC and 193 ethnically matched healthy controls by a semiautomatic fluorescently labeled PCR method. All the subjects were of Chinese Han ethnicity. The frequency of MICB-CA18 was significantly higher in UC patients compared with the healthy controls ($P = 0.0016$) and was increased in the female patients compared with the female healthy controls ($P = 0.0006$). Thus, MICB-CA18 is positively associated with UC patients in the Chinese population[28].

HLA class Ⅱ gene and HLA-DRB1 gene

The HLA region located on chromosome 6p encodes the highly polymorphic, classical class I and Ⅱ genes essential for normal lymphocyte function; it also encodes a further 224 genes. Many early studies investigating this region were limited by small sample size, poor statistical methodology, population stratification and variable disease definition. Although more recent studies have improved study design, investigators are still challenged by the complex patterns of linkage disequilibrium across this gene-dense region, and by the disease heterogeneity characteristic of all genetically complex disorders. Evidence is accumulating that both genetic and environmental factors contribute to UC. The most consistent genetic associations have been shown for the MHC locus HLA class Ⅱ alleles, but the IL-1 families of genes and the multidrug resistance gene MDR1 have also been implicated as genetic susceptibility factors for the development of disease. There is a relationship between UC and bacterial flora, with an increased number of adherent *Bacteroides spp.* and Enterobacteriaceae present in inflamed bowel segments[29].

DRB1 genotype is now thought to act mostly on disease phenotype. The presence of a double dose of RA-associated genes is associated with severe disease with cartilage destruction and increased frequency of extra-articular manifestations. IL-1 is the dominant cartilage-destructive cytokine and its impact on cartilage destruction can be reduced by regulatory cytokines such as IL-4 and IL-10. Increased frequency of particular polymorphism of *IL-1* and *IL-10* genes has been recently identified in the simultaneous presence of susceptible *DRB1* genes, and a specific polymorphism of exon 5 of the *IL-1β* gene is suggested to be predictive of erosive arthritis. Thus, the influence of DRB1 genotype on RA phenotype could be related to genetically controlled patterns of production of cytokines involved in cartilage erosion.

The pathogenesis of UC and CD is still unknown, but the importance of genetic susceptibility has been clearly shown by epidemiological data from family studies. Linkage studies have identified two susceptibility loci for IBD on chromosomes 12 and 16. Importantly, these linkages have been replicated by independent investigators, and studies of positional candidates within these regions continue, together with fine mapping strategies. Regions of suggestive linkage on chromosomes 1, 3, 4, 6, 7, 10, 22 and X have also been reported in individual studies. Other important candidate genes investigated include the IL-1 receptor antagonist, MUC3 and genes of the HLA system. The apparently conflicting data in different studies from around the world may be explained by ethnic differences,

case mix and genetic heterogeneity. Replicated class Ⅱ HLA associations include HLA DRB1*0103 and DR2 (DRB1*1502) involved in UC susceptibility, and HLA DRB1*03 and DR4 as resistance alleles for CD and UC, respectively. Animal studies have provided insights from targeted mutations and quantitative trait locus analysis. The goals of continuing research include narrowing the regions of linkages and analysis of candidate genes, and the application of newly developed methods using SNPs. Advances in IBD genetics hold the potential to provide knowledge about the disease pathogenesis at the molecular level, with ensuing benefits for clinical practice[29-30].

Antigen presentation by MHC class Ⅱ molecules plays an important role in controlling immunity and autoimmunity. Multiple co-factors including the invariant chain (Ii), HLA-DM and HLA-DO are involved in this process. Chen *et al*[30] found that DO inhibits presentation of endogenous self-antigens and that development-regulated DO expression enables antigen-presenting cells to preferentially present different sources of peptide antigens at different stages of development. Disruption of this regulatory mechanism can result in not only immunodeficiency but also autoimmunity. Clinical tests for any of these potential genetic defects are not yet available. They proposed the use of multi-color flow cytometry in conjunction with intracellular staining to detect expression of Ii, DM and DO in peripheral blood B cells, as a convenient reliable screening test to identify individuals with defects in antigen presentation.

Subgroups of UC patients have been further defined by the presence of anti-neutrophil cytoplasmic antibodies (ANCAs). Lee *et al*[31] attempted to define the *HLA class Ⅱ* genes (DRβ, DQα, DQβ) and their relationship with ANCA in southern Chinese patients with UC. Patients were tested for class Ⅱ genes by RFLP and PCR. The indirect immunofluorescence test was used to detect ANCA in the sera. Ethnically matched normal controls were used for comparison. In ANCA-positive UC patients, there was a strong association with the HLA-DQα1c allele ($P < 0.0001$) when compared with controls. This association was not found in ANCA-negative UC patients ($P = 0.21$). In Chinese UC patients, ANCA positivity is associated with the HLA-DQα1c allele, which is not the case in Caucasian patients[31].

Three human mucin cDNAs (Muc-1, Muc-2 and Muc-3) have recently been cloned and sequenced. The major portion of each mucin consists of sequences repeated in tandem along the protein. Three mucins are distinct due to differences in tandem repeat length, lack of sequence homology and different chromosomal locations of their genes. Since altered mucin glycosylation occurs in cancer, resulting in exposure of core carbohydrate, Yuan[32] postulated that increased exposure or other alteration of core peptide structure might occur in cancerous tissues. Antibodies against Muc-1, Muc-2 and Muc-3 tandem repeats were used for immunohistochemical analysis of normal, non-malignant and cancer tissues. The results indicate that, in normal tissues, only Muc-2 was expressed, while in cancerous tissues, all three mucin core peptides were significantly accumulated. All of the three mucin core peptides were increasingly expressed in adenoma, dysplastic epithelium and active UC (pre-malignant lesions), but not in hyperplastic polyps, ischemic colitis and quiescent UC (non-malignant diseases)[32].

The genetic factors predisposing to UC have remained totally unclear to date. HLA-DRB1 genotyping was carried out in 72 unrelated patients with UC and 314 healthy controls using PCR-SSP[33]. All of the patients and healthy controls are Han people in China. The frequency of DRB1*07 allele was increased in UC patients compared with healthy controls ($P = 0.0229$), but the significance disappeared when Bonferroni correction was applied ($P = 0.2977$). Furthermore, compared with healthy controls, although HLA-DRB1*07, DRB1*16/DRB1*09 and DRB1*07/DRB1*12 genotypes were increased in frequency in the patients with extensive colitis, and the patients without extra-intestinal manifestations (EIMs) carried an increased frequency of HLA-DRB1*07 and DRB1*07/DRB1*12 genotypes, although these differences did not reach statistical significance after Bonferroni correction. HLA-DRB1 alleles showed no strong association with UC, and no HLA-DRB1 alleles or genotypes were strongly associated with clinical subgroups of UC in Chinese patients[33].

IL-18 and IL-4 genes

IL-18 is a pro-inflammatory cytokine. Although IL-18 has been implicated as a mediator of antibacterial defense, detrimental effects of IL-18 during bacterial infections have also been demonstrated. Microglia and astrocytes can produce IL-18. *Streptococcus. pneumoniae* is an important microorganism in meningitis. IL-18 plays an important role in sarcoidosis by inducing IFN-γ. The roles of -137 (G/C), -607 (C/A), and -656 (G/T) SNPs of *IL-18* gene promoter regions were compared between 176 individuals in a control group and 161 patients in an experimental group. The major haplotypes-137G/-607C/-656G had a higher promoter activity under the stimulus of sodium butyrate than another major haplotype, -137G/-607A/-656T. This coincided with the genotype with a high IL-18 concentration in the serum. Smokers had a significantly shorter clinical course than non-smokers. A difference in protein expression based on the disparities of *IL-18* gene promoter activity explains the different clinical picture for sarcoidosis, and suggests the effect of smoking on the disease[34].

IL18 was mapped to 11q22.2-22.3 in 1998. Owing to IL-18's important and novel role in immunomodulation, the gene itself has been subject to scrutiny, with the aim of discovering variants that may affect disease susceptibility and/or progression. Despite being sequenced numerous times in different populations, no non-synonymous variants have been found. However, a number of polymorphisms within the proximal promoter have been verified that may interfere with transcription-factor-binding sites. Many of the subsequent association analyses have centered on these

variants, but have yielded no consistent results, despite numerous different study populations being genotyped. IL18 has recently been resequenced in its entirety, enabling the tagging SNP methodology to be adopted. This approach has yielded interesting results, with genetic variation affecting protein levels, and risk. The review by Thompson *et al*[34] aims to compile and reflect the data of interest published to date, with a focus on the diseases related to aberrant inflammatory control.

Under normal situations, the intestinal mucosa is in a state of 'controlled' inflammation regulated by a delicate balance of proinflammatory (TNFα, IFNγ, IL-1, IL-6, IL-12) and anti-inflammatory cytokines (IL-4, IL-10, IL-11). The mucosal immune system is the central effector of intestinal inflammation and injury, with cytokines playing a central role in modulating inflammation. Cytokines may, therefore, be a logical target for IBD therapy using specific cytokine inhibitors. Biotechnology agents targeted against TNF, leukocyte adhesion, Th1 cell polarization, T-cell activation or NF-κB, and other miscellaneous therapies are being evaluated as potential therapies for IBD. In this context, infliximab is currently the only biological agent approved for the treatment of inflammatory and fistulizing CD. Other anti-TNF biological agents have emerged, including CDP 571, certolizumab pegol (CDP 870), etanercept, onercept and adalimumab. However, ongoing research continues to generate new biological agents targeted at specific pathogenic mechanisms involved in the inflammatory process. Lymphocyte-endothelial interactions mediated by adhesion molecules are important in leukocyte migration and recruitment to sites of inflammation, and selective blockade of these adhesion molecules is a novel and promising strategy to treat UC and CD. Therapeutic agents that inhibit leukocyte trafficking include natalizumab, MLN-02 and alicaforsen (ISIS 2302). More controlled clinical trials are currently being conducted, exploring the safety and efficacy of old and new biological agents and the research certainly will open new and exciting perspectives on the development of therapies for IBD.

Eighty-one UC patients and 114 healthy subjects were enrolled by Peng *et al*[35]. *IL-1β*, *IL-1RA* and *IL-4* gene polymorphisms were analyzed with RFLP-PCR and PCR-SSP, respectively. The gene frequency of allele RP2 of IL-4 in patients with UC was significantly higher than that in healthy subjects ($P = 0.00002$), but the gene frequency of allele RP1 in HS was significantly higher than that in UC patients ($P = 0.00002$). The OR of the genotype RP1.2 and RP2.2 was 2.71 and 9.04 respectively. There was no difference in the gene frequencies of IL-1β and IL-1RA between patients with UC and healthy subjects ($P > 0.05$). When patients with UC were divided into ANCA-positive and -negative groups, there was a significant difference in the gene frequencies of allele RP1 and RP2 of IL-4 between the two groups ($P < 0.05$). There is a correlation between the Chinese UC patients and the gene polymorphisms of intron 3 of IL-4. The gene frequency of allele RP1 in UC patients is lower, but the gene frequency of allele RP2 is significantly higher. The differences in gene frequencies of IL-4 between the UC patients and healthy subjects are mainly found in the ANCA-positive UC patients. The Chinese UC patients are not associated with IL-1β and IL-1RA gene polymorphisms[35].

MICA-A5

The role of MICA protein in the immune response is unknown. Recently, it was shown that this polymorphic molecule is mainly expressed by epithelial cells and interacts with the γδT cells. γδT cells appear to dominate lymphocyte populations isolated from epithelium. T lymphocytes bearing γδ receptors have also been isolated from the female genital tract. Expression of MICA by cervical epithelium and its recognition by γδT cells suggest that it may be important in immune surveillance and direct induction of mucosal immunity[35-37]. IBD arises in part from a genetic predisposition, through the inheritance of a number of contributory genetic polymorphisms. These variant forms of genes may be associated with an abnormal response to normal luminal bacteria. A consistent observation across most populations is that any of three polymorphisms of the caspase-activated recruitment domain (CARD15) gene are more prevalent in IBD patients as compared with unaffected controls. Similar aberrant responses to bacteria are associated with variants in autophagy-related 16-like 1 (ATG16L1) and human defensin (HBD-2, -3 and -4) genes. The defective bacterial signal in turn leads to an excessive immune response, presenting as chronic gut inflammation in susceptible individuals. Inconsistent population reports implicate the MHC, which encodes a number of HLA, antigens MICA or cytokines, such as TNFα. Toll-like receptors encoded by the *TLR4* or *TLR9* genes may also play a role. Recent whole genome scans suggest that a rare variant in the IL-23 receptor (IL23R) gene may actually protect against IBD. Other implicated genes may affect mucosal cell polarity (Drosophila discs large homologue5, DLG5) or mucosal transporter function (sodium dependent organic transporters, SLC22A4 and SLC22A5). A variant in ABCB1 (ATP-binding cassette subfamily B member 1) may be especially associated with increased risk of UC. While pharmacogenetics is increasingly being used to predict and optimize clinical response to therapy, nutrigenetics may have even greater potential. In many cases, IBD can be controlled through prescribing an elemental diet, which appears to act through modulating cytokine response and changing the gut microbiota. More generally, no single group of dietary items is beneficial or detrimental to all patients, and elimination diets have been used to individualize dietary requirements. However, recognizing the nature of the genes involved may suggest a more strategic approach. Pro- or prebiotics will directly influence the microbial flora, while immunonutrition, including omega-3 fatty acids and certain polyphenols, may reduce the symptoms of gut inflammation. The expression of gut transporters may be modulated through various herbal remedies, including green tea polyphenols. Such approaches would

require that the gene of interest is functioning normally, other than its expression being up- or down-regulated. However, new approaches are being developed to overcome the effects of polymorphisms that affect the function of a gene. A combination of human correlation studies with experimental models could provide a rational strategy for optimizing nutrigenetic approaches to IBD[36].

MICA plays a role in regulating protective responses by intestinal epithelial Vδ1 γδT cells and the polymorphisms of MICA were reported to be related to several autoimmune diseases. Henckaerts *et al*[11] investigated the association of the microsatellite polymorphisms of TM region of the *MICA* gene with the susceptibility to UC in the Chinese population. The microsatellite polymorphisms of the MICA gene were genotyped in 86 unrelated Chinese patients with UC and 172 ethnically matched healthy controls by a semiautomatic fluorescently labelled PCR method. All the subjects were of Chinese Han ethnicity. The frequency of MICA-A5.1 homozygous genotype and A5.1 allele was significantly increased in UC patients compared with healthy controls ($P = 0.0009$ and $P = 0.0014$). When adjusting for the effects of gender and age at onset, MICA-A5.1 homozygous genotype and A5.1 allele were also increased in the UC patients. Moreover, MICA-A5.1 allele was significantly increased in frequency in the female UC patients ($P = 0.0095$). Logistic regression analysis also revealed that gender was independently associated with UC patients carrying the MICA-A5.1 allele ($P = 0.046$), although the UC patients with extensive colitis ($P = 0.005$) and those with EIMs ($P = 0.0039$) were more likely to carry the MICA-A5.1 allele. EIMs were associated with extent of disease ($P < 0.0001$) and MICA-A5.1 allele was not associated with UC patients with extensive colitis or with EIMs in the logistic regression analysis. Therefore, the MICA-A5.1 homozygous genotype and A5.1 allele were closely associated with UC and the MICA-A5.1 allele was positively associated with the female UC patients in the Chinese population[37].

CD14 and TLR4 genes

TLR and CD14 are components of the lipopolysaccharide receptor complex. A large volume of has been research undertaken on the genetic susceptibility of IBD. Genome-wide linkage studies pointed towards more than 10 chromosomal regions, and fine-mapping of these regions led to the identification of a number of genes, including *CARD15* (*NOD2*), *DLG5*, *OCTN1* and *2*, *TLR4* and *CARD4* (*NOD1*). With the recent completion of the human genome project, whole genome association studies have now become possible and have identified additional genes (*IL23R*, *IRGM*, *PTGER4*, *ATG16L1*) for CD and UC, which have subsequently been replicated. At present, the CARD15 gene is still the most understood susceptibility gene, explaining around 20% of the genetic predisposition to CD. Prediction of disease phenotype and response to the main therapies has for many years been a goal for physicians treating IBD patients. Only now, we have started to accumulate some evidence proving that genetic factors indeed influence both the clinical course of IBD patients and their likelihood of responding to certain therapies. In the coming years, we expect an exponential increase in the efforts devoted to research in this area. The optimal prediction of both disease behavior and response to therapy might result from complex combinations of clinical, biochemical, serological and genetic factors[38].

RFLP-PCR was used to genotype polymorphisms TLR4 Asp299Gly and CD14 C-260T in 114 patients with UC and 160 healthy controls in the Chinese Han population. Moreover, a comparison was made with 170 healthy Dutch white subjects[39]. No TLR4 Asp299Gly mutation was detected in any patients or healthy controls in the Chinese Han population, which was similar to Japanese subjects, but the mutation occurred in 10% of the Dutch white subjects. There were no significant differences of CD14 genotypes between healthy controls and the patients in Chinese patients with UC.

Fas-670 gene

Fas (Apo-1/CD95) antigen is a 45-kDa type I membrane protein, which is expressed in various tissues and cells. Fas is a member of the TNF superfamily and mediates apoptosis when cross-linked with agonistic anti-Fas antibody or Fas ligand (FasL). Although the best-characterized physiological system involving Fas/FasL-mediated apoptosis is observed in the immune system, a role of Fas/FasL in non-lymphiod tissues has become increasingly evident. Fas-mediated apoptosis is thought to be involved in autoimmune disease and inflammatory disorders. Recent studies have suggested that immune dysregulation and genetic factors play important roles in the pathogenesis of IBD. Defective apoptosis of lamina propria T cells may be a factor in mucosal immune dysregulation and tissue inflammation. One of these polymorphisms is a single nucleotide substitution at the -670 position that alters the *Mva* I restriction enzyme cutting site, creating an RFLP. This polymorphism is situated at the consensus sequence site, the gamma interferon activation site. This site can bind to transcription factors such as STATs, thus exerting an effect on the level of transcription of the Fas protein. Although expression and functional effects of the Fas antigen have been found to be associated with IBD, the relationship between Fas-670 polymorphism and IBD has not been reported yet. In a recent study, Peng *et al*[35] could not find any significant association between Fas-670 polymorphism and IBD, which indicates genetic heterogeneity of the diseases. Since Fas-670 polymorphism does not contribute to IBD, there may be other genes that are involved in the pathogenesis of IBD, and other mechanisms of gene regulation may influence Fas-mediated epithelial apoptosis in IBD.

For the Fas-670 polymorphism, it has been hypothesized that either increased apoptosis of intestinal epithelium or decreased apoptosis of lamina propria lymphocytes may induce inflammation of the gut. Fifty unrelated Chinese patients with IBD (38 patients with UC and 12 with CD) and 124 healthy controls were

genotyped for the Fas-670 polymorphism by RFLP-PCR. The PCR product was digested by *Mva* I restriction enzyme. Distribution of the Fas-670 gene polymorphism was 33% for the AA genotype, 52% for the AG genotype and 15% for the GG genotype in 124 healthy subjects, and 30% for the AA genotype, 42% for the AG genotype and 28% for the GG genotype in patients with IBD. However, there was no significant difference in the genotype (P = 0.1498), allele frequencies (P = 0.3198) and carriage frequencies (P = 0.4133) between healthy controls and IBD patients. Furthermore, no difference was found between left-sided and total colitis (P = 0.8242). Fas-670 polymorphism is not associated with IBD in Chinese patients. In a recent study, Xia *et al*[40] genotyped Fas-670 polymorphism in Chinese patients with IBD and healthy controls, and found that the polymorphism was not associated with UC and CD. The study suggested that Fas-670 polymorphism might not play a role in susceptibility of IBD in Chinese patients.

p53 gene

The *p53* gene like the *Rb* gene is a tumor suppressor gene, i.e. its activity stops the formation of tumors. If a person inherits only one functional copy of the *p53* gene from their parents, they are predisposed to cancer and usually develop several independent tumors in a variety of tissues in early adulthood. This condition is rare, and is known as Li-Fraumeni syndrome. However, mutations in p53 are found in most tumor types, and so contribute to the complex network of molecular events leading to tumor formation. The *p53* gene has been mapped to chromosome 17. In the cell, p53 protein binds DNA, which in turn stimulates another gene to produce a protein called p21 that interacts with a cell division-stimulating protein (Cdk2). When p21 is combined with Cdk2, the cell cannot pass through to the next stage of cell division. Mutant p53 can no longer bind DNA in an effective way, and as a consequence the p21 protein is not made available to act as the stop signal for cell division. Thus cells divide uncontrollably, and form tumors.

p53 protein expression was detected by immunohistochemistry in 70 specimens from 21 cases of UC and 25 colonic mucosa specimens from normal subjects. The specimens of UC were examined for the mutation in exon 5, 6, 7, 8 of *p53* gene with the microdissection-PCR-SSCP/HA-clone-sequencing technique and the alterations in 10 microsatellite loci with the microdissection-PCR-SSLP-clone-sequencing technique[41]. None of 25 normal specimens was p53-positive immunohistochemically, while 4/21 of UC specimens were p53-positive. p53-positive rate in inflammatory mucosa of UC specimens was 0/5, and 1/7, 2/7 and 1/2 in low-grade displasia (LGD), high-grade displasia (HGD) and carcinoma, respectively. The abnormal exons were detected by SSCP and confirmed by sequencing in two out of 21 cases: one was exon 6 in a case with carcinoma and the other was exon 8 in an HGD case; both had positive p53 expression. Two cases were positive in the Bat26 locus by SSLP: one was an LGD case, and the other was a case of carcinoma, which also had abnormal exon 6 of *p53* gene. Another nine microsatellite loci, [TGFβRII (A) (10), IGFIIR (G) (8), IGFIIR (CT) (5), TGFβRII (GT) (3), BAX (G) (8), hMSH3 (A) 8, hMSH6 (C) (8), TCF4 (A) (9) and DPC4 (CA) (17)] were negative in all cases. The *p53* gene mutations and microsatellite instability may be one of the mechanisms for higher risk of carcinogenesis in UC[42].

NF-κB

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that acts as a transcription factor. NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens. NF-κB plays a key role in regulating the immune response to infection. Consistent with this role, incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development. NF-κB has also been implicated in the processes of synaptic plasticity and memory.

In the healthy gut, the mucosal immune system ensures the balance between pro- and anti-inflammatory mediators, thereby allowing an effective defense against luminal pathogens, but at the same time prevents an overwhelming immune reaction directed against the huge amount of harmless luminal antigens (e.g. components of food or non-pathogenic bacteria). In both entities of IBD (CD and UC), this immunological balance is severely impaired and shifted towards the pro-inflammatory side. The chronic mucosal inflammation in IBD is caused by hyperactivation of effectors immune cells, which produce high levels of pro-inflammatory cytokines like TNFα, IL-6 and IFNγ, resulting in colonic tissue damage. NF-κB was identified as one of the key regulators in this immunological setting. Its activation is markedly induced in IBD patients, and through its ability to promote the expression of various pro-inflammatory genes, it strongly influences the course of mucosal inflammation. Considering the different cell-type specific effects that are mediated by NF-κB, the authors described its complex role in IBD and discussed the existing pharmacological attempts to block the activation of NF-κB to develop new therapeutic strategies in IBD.

A total of 27 cases of UC were investigated. Fifteen cases received sulfasalazine (SASP) treatment or SASP and glucocorticoid treatment, and 12 patients did not receive any medication related to UC[41]. Normal mucosa from nine colon cancer patients served as a control. Ten pieces of intestinal mucosal biopsy specimens were obtained from each patient. The mRNA expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were determined by RT-PCR. The protein levels of ICAM-1 and VCAM-1 were measured by ELISA. NF-κB DNA binding activity was evaluated by electrophoretic mobility shift assay (EMSA). The results showed that NF-κB DNA binding activity, mRNA and protein expression of

ICAM-1 and VCAM-1 were increased significantly in patients with UC compared with normal control ($P < 0.05$). Glucocorticoids and SASP markedly inhibited NF-κB activation and significantly decreased mRNA and protein expression of ICAM-1 and VCAM-1 ($P < 0.05$). Adhesive molecule (ICAM-1 and VCAM-1) gene activation had significant positive correlation with the NF-κB DNA binding activity ($P < 0.05$, $P < 0.05$, respectively). NF-κB is a major and essential factor in regulating the expression of adhesive molecules; it plays an important role in the pathogenesis of UC. SASP and glucocorticoids ameliorate UC *via* inhibition of NF-κB activation and reduction of adhesive molecule expression[42].

Among the 31 patients with UC, 17 patients received SASP or SASP and glucocorticoid treatment, and 14 patients did not receive any medication related to UC. Normal mucosa from 11 colon cancer cases served as a control. Ten pieces of intestinal mucosal biopsy specimens were obtained from each patient. NF-κB DNA binding activity was evaluated with EMSA. Expression of cytokine mRNA was studied RT-PCR. The expression of IL-1β and IL-8 mRNA was increased significantly in patients with UC, as compared with that in the control specimens ($P < 0.05$), and had a significant positive correlation with NF-κB DNA binding activity ($P < 0.05$, $P < 0.05$, respectively). Glucocorticoids and SASP strongly inhibited NF-κB activation and significantly decreased the expression of IL-1β and IL-8 mRNA. NF-κB is a major and essential factor in regulating the expression of cytokine and plays a fundamental role in the pathogenesis of UC. SASP and glucocorticoids decreased cytokine expression *via* inhibition of NF-κB activation[43].Various components of the mucosal immune system are implicated in the immunopathogenesis of UC. Evidence from animal models also suggests that an altered immune response to the commensal microflora of the host plays a central role in the development of UC. Therefore, it is elucidated that the cells and molecules are implicated in the immunopathogenesis of the disease from four aspects: antigens in the intestine, dendritic cells, TLRs and NF-κB in UC[44].

Ten pieces of colon mucosal biopsy specimens were obtained from 31 patients with UC, 17 of whom received SASP or SASP plus glucocorticoid and 14 received no medication. Samples of normal mucosa around the lesion taken from 11 patients with colon cancer were used as controls. NF-κB DNA binding activity was evaluated by EMSA. NF-κB p65 expression was determined by Western blot analysis and immunohistochemical staining with a NF-κB p65 antibody. The type of cells containing activated NF-κB p65 was identified by double immunofluorescence confocal laser scanning microscopy. The expression of NF-κB p65 and NF-κB DNA binding activity was significantly higher in patients with UC than in the controls ($P < 0.05$), and was correlated with the degree of inflammation. The NF-κB expression was significantly stronger in the nuclei than in the cytoplasm in patients with UC without pharmacotherapy. The NF-κB expression in nuclei was significantly stronger in the group without pharmacotherapy than in the group with pharmacotherapy ($P < 0.05$). Only a few NF-κB p65-positive cells were seen in the controls. NF-κB p65 expression was found in all major subsets of mononuclear cells, including macrophages, B lymphocytes, T lymphocytes, and cryptal epithelial cells. The increased activation of NF-κB and increased expression of NF-κB may be involved in the pathogenesis of UC. Glucocorticoids and SASP strongly inhibited NF-κB activation and expression. The inhibition of NF-κB activation may be a central part of the anti-inflammatory action of glucocorticoids and SASP, which might represent an important pharmacological mechanism in the treatment of patients with UC. NF-κB will be an important target for cytokine-based therapy of UC[45].

Table 1 Epidemiology and gene markers of IBD[2-6,48,49]

Epidemiology	Gene markers of IBD
Incidence per 100 000	0.5-2.0 (China); 2-14 (North America)
Prevalence per 100 000	1-23 (China); 26-246 (North America)
Geography	Northern Countries > Southern Countries; Lower (China)
Age of onset (yr)	20-35 (China)
Sex	M > F (China)
Race	Whites > Blacks; Lower (Chinese)
Possible genetic associations (Chinese)	*TNF-308A*, *CARD15* (*NOD2*), *MIF-173*, NAT2, *NKG2D*, *STAT6*, *CTLA-4*, *MICA-MICB*, *HLA-DRB1*, *HLA class Ⅱ*, *IL-18*, *IL-4*, *MICA-A5*, *CD14*, *TLR4*, *Fas-670*, *p53*, *NF-κB*; Chromosome 3, 5, 7, 12, 16 ,19

CONCLUSION

Epidemiology and genetic research in IBD (UC and CD) has provided knowledge about the complexity and heterogeneity of the disease and has 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 development of better new drugs and therapies. The most important advance toward understanding this process has been identification of specific genetic associations with IBD, which will shed new light on future research of IBD. Researchers are studying how and why the immune system is activated, how it damages the colon, and the processes involved in healing. Currently, numerous clinical trials on UC are being conducted. Immunomodulators used for treating severe UC include azathioprine/6-MP, methotrexate and cyclosporine. Integrated traditional Chinese and modern medicine is safe and effective in maintaining remission in patients with UC[1,11,46]. There are also complementary and alternative therapies for IBD[47]. Epidemiology and gene markers of IBD are shown in Table 1.

UC and CD are complex polygenic disorders, characterized by several genes, together with environmental factors contributing to the development of IBD. Recent advances in research on genetic susceptibility have allowed the identification of diverse genes at different levels, innate immunity, antigen presentation molecules,epithelial integrity, drug transporters and cell adhesion. The application of genetic testing into clinical practice has become available and all genetic markers may have several clinical implications: prediction of disease phenotype, molecular classification, prevention of complications, and prognosis.

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TOPIC HIGHLIGHT

Jose JG Marin, Professor, *Series Editor*

Bile acids: Chemistry, physiology, and pathophysiology

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Abstract

The family of bile acids includes a group of molecular species of acidic steroids with very peculiar physical-chemical and biological characteristics. They are synthesized by the liver from cholesterol through several complementary pathways that are controlled by mechanisms involving fine-tuning by the levels of certain bile acid species. Although their best-known role is their participation in the digestion and absorption of fat, they also play an important role in several other physiological processes. Thus, genetic abnormalities accounting for alterations in their synthesis, biotransformation and/or transport may result in severe alterations, even leading to lethal situations for which the sole therapeutic option may be liver transplantation. Moreover, the increased levels of bile acids reached during cholestatic liver diseases are known to induce oxidative stress and apoptosis, resulting in damage to the liver parenchyma and, eventually, extrahepatic tissues. When this occurs during pregnancy, the outcome of gestation may be challenged. In contrast, the physical-chemical and biological properties of these compounds have been used as the bases for the development of drugs and as pharmaceutical tools for the delivery of active agents.

INTRODUCTION

Over the last decades the interest of hepatologists in bile acids has grown markedly[1]. The reason has been the discovery of the role of these acidic steroids in many different physiological processes, which has important implications from the point of view of liver and intestinal pathology and pharmacology. Moreover, in recent years their use in supramolecular chemistry, materials chemistry and nanotechnology has been the focus of intensive research[2]. Bile acids include a group of molecular species with similar, but not identical, chemical structures. Surprisingly, they exhibit diverse physical properties and even more divergent biological characteristics. Although their best-known role is their participation in the digestion and absorption of fat, they play an important role in several other functions. In the present review, these roles will only be mentioned briefly because they are addressed in depth in other reviews of this series. The relevance of their physiological roles explains why genetic abnormalities accounting for alterations in their synthesis, biotransformation and/or transport may result in severe alterations, even leading to lethal situations, for which, in pediatric patients, the sole therapeutic option may be liver transplantation.

Moreover, the increased levels of bile acids that may

be reached during cholestatic liver diseases are known to induce oxidative stress and apoptosis that results in damage to the liver parenchyma and, eventually, extrahepatic tissues. When this occurs during gestation, such as in women suffering from intrahepatic cholestasis of pregnancy, the outcome of the gestational process and/or the health of the fetus may be challenged. These aspects will be also be considered in depth in a separate review of this series.

In contrast to the involvement of bile acids in the etiology and pathogenesis of several diseases, the physical-chemical and biological properties of these compounds have permitted them to be used in the development of drugs and as pharmaceutical tools for the delivery of active agents, as will be commented below.

PHYSICAL-CHEMICAL CHARACTERISTICS OF BILE ACIDS

Chemical structure

In the common biomedical literature, the terms "bile acids" or "bile salts" are generally used to denote the so-called "modern" bile acids[3]. They have 24 carbon atoms and are abbreviated as C_{24} bile acids, in contraposition to "primitive" bile acids, which have 25-27 carbon atoms (C_{27}, C_{26}, C_{25} bile acids) and are present in the bile acid pool of primitive (e.g. coelacanth and sharks) and less primitive (e.g. reptiles and amphibians) vertebrates. The structures of some of the most abundant bile acids in humans are depicted in Figure 1. In higher vertebrates, C_{24} bile acids constitute a major part of the bile[4], and in human bile, these compounds are almost completely in conjugated form with either glycine (75%) or taurine (25%)[5]. Under physiological conditions, conjugation increases their water-solubility.

Bile salts have a unique and fascinating molecular structure derived from a saturated tetracyclic hydrocarbon perhydrocyclopentanophenanthrene system, usually known as the steroid nucleus. The steroid nucleus is also the main carbon skeleton of other families of compounds such as brassinosteroids, ubiquitously distributed throughout the plant kingdom[6], hopanoids, commonly used as biomarkers in organic geochemistry[7], triterpenoids[8], and hormones.

The steroid nucleus consists of three six-member rings (A, B and C) and a five-member ring (D), with a curved (beaked) or flat structure (depending on a *cis*- or *trans*-fused configuration between the A and B rings). In mammals, the nucleus is almost invariably 5β (A/B junction in *cis* configuration), while in lower vertebrates, some bile acids, known as *allo*-bile acids, exhibit an A/B *trans*-fusion. There are 11 chiral carbon atoms. Bile acid molecules are approximately 20 Å long, with an average radius of about 3.5 Å (Figure 2).

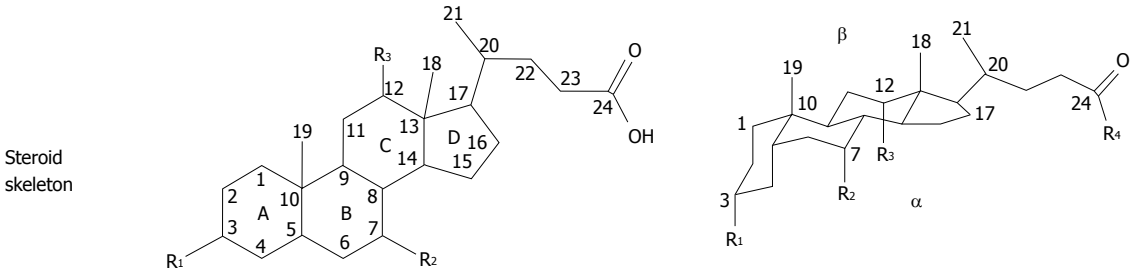
As early as the 1960s, Haslewood had noticed the biological significance of chemical differences in bile salts[9] and that the chemical nature of the bile salts of more primitive animals clearly indicates that an evolution from C_{27}, 5α-alcohol sulfates to C_{24}, 5β-acids has taken place[10]. Bile acids from different species differ chemically in three structural aspects: (1) side-chain structure; (2) stereochemistry of the A/B ring fusion (as mentioned above); and (3) the distribution of the number, position and stereochemistry of hydroxyl groups in the steroid nucleus. Nearly all primary bile acids and bile alcohols, which occur in the less evolved forms of life, have a 7α-hydroxyl group; ursodeoxycholic acid (UDCA) being a notable exception. Most evolved mammalian bile acids have a 5β-configuration with hydroxyl groups at 3α, 7α and 12α, whereas C_{27} bile alcohol sulfates (which increases water solubility) are widespread in nature. These latter are the dominant bile salts of ancient mammalian species, cartilaginous fishes, and some amphibians. The West Indian manatee was the first mammal found to lack bile acids, presumably because it lacks the enzymes required for oxidation of the 26-hydroxy group to a carboxylic acid[11].

Physical characteristics

The presence in bile acid molecules of chemically "non-equivalent" hydroxyl groups (in mammals, commonly at positions 3, 7 and/or 12) and the side chain structure supporting a carboxylic acid group confer them peculiar physical-chemical characteristics, which has made them very attractive building blocks, with repercussions in the design of novel antibiotics[12-14], chiral templates[15], new soft materials[16,17], cation[18] and anion[19,20] receptors, artificial ion channels[21], drug targeting vehicles[22], dendrons[23], molecular baskets[24], scaffolds for combinatorial chemistry[25], new surfactants[26], and others[27,28].

Among the most important physiological properties of bile salts, lipid transport by solubilization and the excretion of cholesterol into the intestinal tract, from which it is poorly absorbed, can be mentioned. These properties are related to their amphipathic nature, which is due to the existence of a hydrophilic side (α-face, concave lower side) and a hydrophobic side (β-face, convex upper side). The hydroxyl groups, oriented towards the α-side (with the exception of the naturally occurring UDCA), and the carboxylic side chain afford them their hydrophilic character. The hydrophobic methyl groups (at C-18 and C-19) are oriented towards the β-side (Figure 1)[29]. As a consequence, they exhibit a great surface activity and in aqueous solutions, they form small aggregates or micelles of usually less than 10 monomers, as long as their concentrations are above a critical value, generally called the critical micellar concentration (CMC). Below the CMC, bile salts behave as 1:1 strong electrolytes, as has been demonstrated from freezing-point measurements[30,31].

The balance between hydrophobic and hydrophilic characters differs markedly among the several molecular species of bile salts. Differences in this balance might account for differences in how bile salts interact with other substances such as, for instance, in the solubilization of phospholipids, cholesterol and other lipids. Over 50 methods have been employed in the



Name	R_1	R_2	R_3	R_4
Cholanic acid	H	H	H	OH
Cholic acid	OH	OH	OH	OH
Chenodeoxycholic acid	OH (α)	OH (α)	H	OH
Deoxycholic acid	OH	OH	OH	OH
Ursodeoxycholic acid	OH (α)	OH (β)	H	OH
Lithocholic acid	OH	H	H	OH
Glicocholate	OH	OH	OH	$NHCH_2COO^-$
Taurocholate	OH	OH	OH	$NHCH_2CH_2SO_3^-$

Figure 1 Structures of the most abundant bile acids in humans, and their glycine and taurine conjugates.

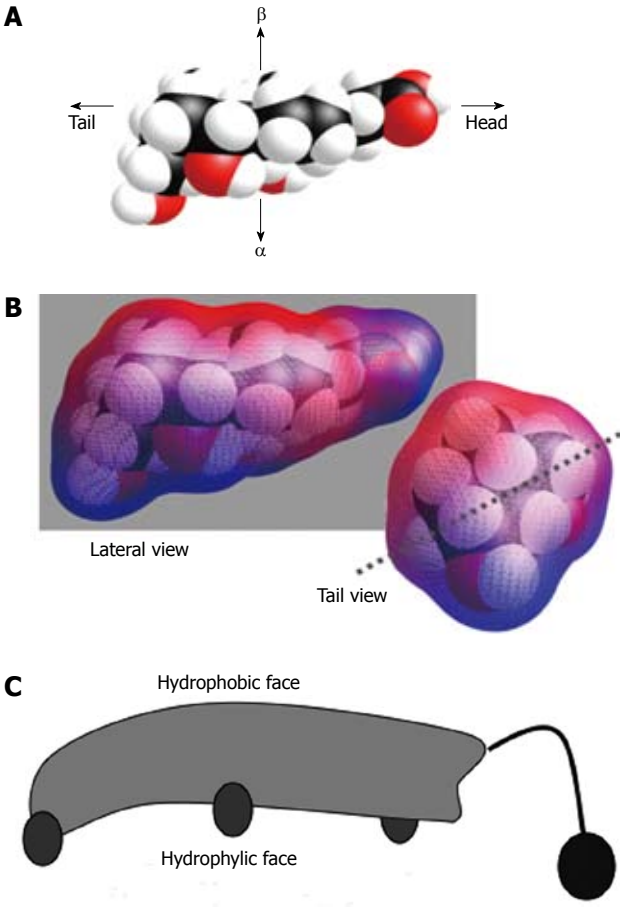


Figure 2 Stereostructure of cholic acid. A: Space-filling model; B: Calculated molecular lipophilic potential[147]. Blue colour shows polar surface and red colour shows apolar surface; C: Cartoon representation (as introduced by Small[148]).

literature to determine the CMC (or pseudo-cmc) values of bile salt solutions, such as the HPLC retention time[32], which accounts in part for the wide range of published values for the CMC[33,34]. The hydrophilicity of the common free and conjugated bile salts decreases in the order UDCA > cholic acid (CA) > chenodeoxycholic acid (CDCA) > deoxycholic acid (DCA) > lithocholic acid (LCA), and taurine-conjugated > glycine-conjugated > free species[35].

These values have been used to predict the cholesterol-solubilizing capacity of all bile salt species, but other physical-chemical and biological properties of individual bile salts also may reflect their hydrophilic-hydrophobic balance[36]. The degree of calcium binding follows the order UDCA < CA < CDCA < DCA < LCA, and taurine-conjugated < glycine-conjugated < free bile salts[35]. In model biles with added gallstones, gallstone masses decrease by addition of bile acids in different degrees, depending on bile acid hydrophobicity (TUDCA > TCA > TCDCA)[37]. However, as noted by Heuman[36], the application of the hydrophilic-hydrophobic balance to determine the physiological properties of bile acids is still an area of controversy. In this respect, Heuman defined a hydrophobic index and extended the method to mixed bile salt solutions[36].

Natalini *et al*[38] have correlated CMC values with hydrophobicity indices, which were determined chromatographically by extrapolating the retention factors back to a virtual pure water-containing mobile phase. Computational methods can also be employed to predict the hydrophobic/hydrophilic balance of bile salts[39]. This balance can be modified by attaching appropriate substituents that enhance either the hydrophilicity or the hydrophobicity of the bile acid, depending on the nature of the organic group. These modifications may be of biological importance. For instance, a series of hydroxycholan-24-amines have been synthesized by modification of the carboxyl group of unconjugated bile acids into a basic moiety[40]. These

Table 1 Minimum and maximum values of CMC in water at 37℃ (in mmol/L) for the sodium salts of major bile acids

Bile acid	Minimum CMC	Maximum CMC
Cholic acid	2.5	29.3
Deoxycholic acid	0.8	70
Chenodeoxycholic acid	3.0	30
Taurocholic acid	1.5	12
Taurodeoxycholic acid	0.6	12
Taurochenodeoxycholic acid	1.25	8

compounds show differential antimicrobial activity against several strains and against fungi[41]. Table 1 summarises the lowest and highest values of CMC reported for the most common bile acids in human bile[33].

PHYSIOLOGY OF BILE ACIDS

Biological functions

Traditionally considered as digestive molecules whose main function is to help in the emulsion and absorption of dietary fats and liposoluble vitamins, bile acids are beginning to be considered more versatile molecules than previously believed. Recent findings have suggested the participation of bile acids in many different functions.

The secretion of bile acids into bile canaliculi generates an osmotic pressure that accounts for the so-called bile-acid-dependent fraction of bile flow[42]. Bile acids stimulate biliary lipid secretion[43] and, due to their physical-chemical properties, are able to form mixed micelles together with biliary phospholipids, which allows the solubilization in bile of cholesterol and other lipohilic compounds. Mixed micelles also account for the emulsion of dietary fat and liposoluble vitamins in the gut, thus helping their absorption. Bile acids also facilitate intestinal calcium absorption[44]. At the intestinal level, bile acids are known to modulate pancreatic enzyme secretion and cholecystokinin release[45]. Moreover, they are potent antimicrobial agents that prevent bacterial over-growth in the small bowel[46].

In the last decade, with the discovery of a specific nuclear receptor able to respond to bile acids, such as the "farnesoid X receptor" (FXR)[47-49], and more recently of their membrane receptor TGR5[50,51], the role of bile acids as signaling molecules with important paracrine and endocrine functions has become evident[52]. Apart from the regulation of their own hepatic synthesis and hepatic and intestinal transport, bile acids are involved in triggering the adaptive response to cholestasis and other insults to the liver[53-55]. Finally, their role in the control of general energy-related metabolism, and more precisely in hepatic glucose handling, has been reported[56].

Synthesis

Bile acids are synthesized from cholesterol (Figure 3). Two main biosynthetic pathways, the so-called "classical" and "alternative" pathways, account for bile acid formation, although several other minor routes have been described, which in some species and situations may also have relevance[57].

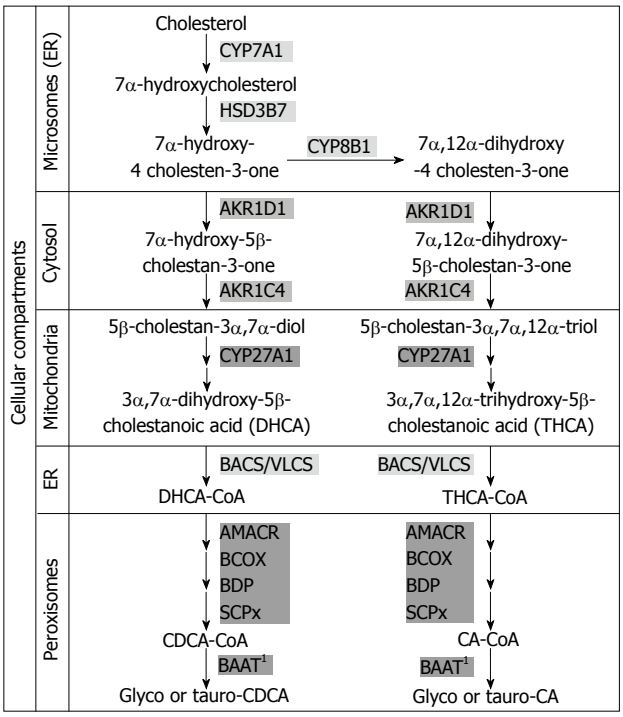


Figure 3 Schematic representation of bile acid synthesis by the classical neutral pathway. AKR1C4: 3α-hydroxysteroid dehydrogenase; AKR1D1: Δ4–3-oxosteroid-5β-reductase; AMACR: Alpha methylacyl-CoA racemase; BAAT: Bile acid; CoA: Amino acid N-acyltransferase ([1]A minor cytosolic fraction does also exist); BACS: Bile acid CoA synthetase; BCOX: Branched-chain acyl CoA oxidase; BDP: D-bifunctional protein hydratase; CYP27A1: Sterol 27-hydroxylase; CYP7A1: Cholesterol 7α-hydroxylase; CYP8B1: Sterol 12α-hydroxylase; HSD3B7: 3β-hydroxy-Δ5-C27-steroid dehydrogenase/isomerase; SCPx: Sterol carrier protein X; VLCS: Very long-chain acyl CoA synthetase; ER: Endoplasmic reticulum.

The classical pathway, also known as the "neutral" pathway because its intermediate metabolites are neutral sterols, is present only in the liver and synthesizes the two primary bile acids in humans: CA and CDCA. This route consists of a cascade of reactions catalyzed by enzymes located at the cytosol, microsomes, mitochondria, and peroxisomes (Figure 3). Extensive descriptions of these reactions and enzymes can be found in several recent reviews[58,59].

In the neutral pathway, the modification of the sterol nucleus of cholesterol precedes the oxidative cleavage of its side chain. It begins with the hydroxylation of cholesterol at C-7, catalyzed by microsomal cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme of the pathway, a cytochrome P450 enzyme localized exclusively in the liver. The resulting 7α-hydroxycholesterol is converted to 7α-hydroxy-4 cholesten-3-one by 3β-hydroxy-Δ5-C27-steroid dehydrogenase/isomerase (HSD3B7), which is also microsomal. The synthesis of CA requires the hydroxylation of 7α-hydroxy-4-cholesten-3-one at the C-12 position, performed by sterol 12α-hydroxylase (CYP8B1), another highly regulated microsomal enzyme[60].

The next steps are catalyzed by two cytosolic enzymes, Δ4–3-oxosteroid-5β-reductase (AKR1D1) and 3α-hydroxysteroid dehydrogenase (AKR1C4), that carry out the reduction of the double bond to obtain 5β-cholestan-3α,7α-diol or 5β-cholestan-3α,7α,12α-triol, the precursors of CDCA and CA, respectively. Mitochondrial sterol 27-hydroxylase (CYP27A1) then oxidizes the side-chain of these precursors by introducing a hydroxyl group to the C-27 position, which is subsequently oxidized to an aldehyde and then to a carboxylic acid. The products, 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) and 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA), respectively, are activated to their coenzyme A-esters by either bile acid CoA synthetase (BACS) or very long chain acyl CoA synthetase (VLCS), both localized at the endoplasmic reticulum. The resulting cholestanoyl-CoAs are then transported into peroxisomes where the side-chain is shortened by β-oxidation, a process that involves the action of four peroxisomal enzymes (Figure 3).

The final step in bile acid synthesis involves conjugation of the terminal side-chain carboxylic acid with the amino acids glycine or taurine, carried out by the enzyme bile acid CoA: amino acid N-acyltransferase (BAAT). BAAT has been reported to be localized both in peroxisomes and in the cytosol[61], suggesting that peroxisomal BAAT is responsible for conjugation of the newly formed primary bile acids within the peroxisome, while cytosolic BAAT may be involved in the re-conjugation of recycled primary and secondary bile acids previously deconjugated by intestinal bacteria. However, recent studies support the notion that BAAT is mainly a peroxisomal enzyme present in undetectable amounts in the cytosol, and hence deconjugated bile acids returning to the liver need to shuttle to the peroxisome to be re-conjugated[62].

In the alternative biosynthetic pathway for bile acids, side-chain oxidation of cholesterol precedes steroid ring modification. Thus, acidic intermediate metabolites are formed and this pathway is also known as the "acidic" pathway. The first step involves the oxidation of cholesterol to 27-hydroxycholesterol by sterol 27-hydroxylase (CYP27A1), followed by conversion into 7α,27-dihydroxycholesterol by oxysterol 7α-hydroxylase (CYP7B1), a microsomal enzyme specific for this acidic pathway. Since both CYP27A1 and CYP7B1 are expressed in various tissues, and because only the liver has all the required enzymes to accomplish bile acid biosynthesis, these oxidized sterols must be transported to the liver in order to be converted to bile acids. In this pathway, CDCA is the main bile acid formed. The relative contribution of the alternative pathway to overall bile acid synthesis depends on the species considered. In humans, it contributes little to the restitution of daily loss of bile acid (approximately 10%) under normal conditions, but may become the major bile acid biosynthetic pathway in patients with liver diseases[63].

Cholesterol can also be oxidized to 25-hydroxycholesterol and 24-hydroxycholesterol, mainly in extrahepatic tissues such as the brain, an organ with a very high expression of sterol 24-hydroxylase (CYP46A1)[64]. The contribution of these other hydroxylase pathways to overall bile acid synthesis is minor. However, biologically active oxysterols are potent regulators of cholesterol metabolism *via* their nuclear receptor; i.e. the liver X receptor (LXR)[65].

Regulation of bile acid synthesis

Bile acids exert a negative feedback regulation on their own synthesis, in particular by inhibiting CYP7A1 activity[66] and expression[67]. In fact, the cytochrome P450 enzymes CYP7A1, CYP8B1 and CYP27A1 involved in bile acid synthesis are subject to negative feedback regulation by bile acids, which is mainly mediated through the nuclear bile acid receptor FXR. Upon activation by hydrophobic bile acids such as CDCA[68], FXR induces the expression of the small heterodimer partner (SHP) transcriptional repressor. SHP in turn negatively interacts with other transcription factors, liver receptor homolog-1 (LRH-1) and hepatocyte nuclear factor-4α (HNF-4α), that bind to the bile-acid response elements (BAREs) located within the promoter region of the CYP7A1 and CYP8B1 genes[69,70], thus resulting in repression of bile acid synthesis[71,72]. Another FXR-dependent but SHP-independent mechanism for bile acid-induced CYP7A1 down-regulation has been described, involving the secreted fibroblast growth factor 19 (FGF-19) and its receptor FGFR4[73]. Recent studies using liver-specific knock-out mice for FXR and LRH-1 provide strong evidence regarding the importance of the FGF-19/FGFR4 pathway in the control of bile acid synthesis[74,75].

Cholesterol modulates its own catabolism to bile acids, mostly at the transcriptional level. Thus, oxysterols activate LXR, which in turn up-regulates CYP7A1 expression in rat hepatocytes. However, LXR has little or no effect on human CYP7A1[76,77] owing to the lack of an LXR-response element in the promoter of the human *CYP7A1* gene.

Hormones and exogenous compounds may also affect bile acid synthesis. Insulin down-regulates several enzymes of the biosynthetic pathway, such as CYP7A1 and CYP27A1, in different animal species[78], although a dual effect has been described in human hepatocytes[79]. Thyroid hormones induce CYP7A1 gene transcription in rats[80], but the effect of thyroid hormones on the regulation of CYP7A1 in humans is still controversial[81]. Regarding the effects of drugs on bile acid synthesis, both phenobarbital, acting through the nuclear receptor constitutive androstane receptor (CAR)[82], and the antibiotic rifampicin, acting through the pregnane X receptor (PXR)[83], have recently been shown to repress CYP7A1 transcription.

Finally, the activity of CYP7A1 undergoes diurnal variations, paralleled by variations in protein and mRNA levels[84]. Recently, it has been shown that HNF-4α is essential for the maintenance of the diurnal variations in CYP7A1 expression[85]. Also, the circulating levels of FGF-19, which participates in the negative regulation of CYP7A1 expression, show a pronounced diurnal

variation in marked synchronicity with the changes in CYP7A1 activity[86].

Biotransformation

During their intestinal transit, bile acid molecules undergo modifications due to the action of intestinal bacteria. The bile acid metabolism by small intestine microbes consists mainly of de-conjugation and hydroxyl group oxidation. Although ileal bile acid absorption is a very efficient process, some of these molecules (< 1 g/d) escape it and enter the large bowel. The major bile acid modifications in human colon include 7α-dehydroxylation, deconjugation, and oxidation/epimerization of hydroxyl groups at C-3, C-7 and C-12. The deconjugation and oxidation reactions are carried out by a broad spectrum of intestinal anaerobic bacteria. In contrast, bile acid 7α-dehydroxylation is restricted to a limited number of anaerobes representing a small fraction of the total colonic flora[87].

Dehydroxylation at position C-7 is quantitatively the most important bacterial bile acid biotransformation event occurring in the human colon. Bacterial dehydratases of the anaerobic flora from this region attack and remove the hydroxyl group to form 7-deoxy bile acids. Thus, the secondary bile acids DCA (3α,12α-dihydroxy-5β-cholanoic acid) and LCA (3α-hydroxy-5β-cholanoic acid) are formed from CA and CDCA, respectively.

On their side chain, bile acids undergo deconjugation, i.e. enzymatic hydrolysis of the C-24 N-acyl amide bond linking bile acids to their amino acid conjugates. Bile salt hydrolases (BSHs) from the choloylglycine hydrolase family form unconjugated bile acids and free glycine or taurine. Some of these molecules of unconjugated bile acids are taken up by the intestine and return to the liver *via* the portal vein, where they are efficiently taken up and reconjugated during their transit across the hepatocytes toward the bile.

The oxidation and epimerization of the 3-, 7- or 12-hydroxyl groups of bile acids are carried out by the hydroxysteroid dehydrogenases (HSDHs) of intestinal bacteria. Epimerization of bile acid hydroxyl groups is a reversible change in stereochemistry from the α to the β configuration (or *vice versa*), with the generation of a stable oxo-bile acid intermediate. The epimerization of CDCA is the origin of the UDCA (3α,7β-dihydroxy-5β-cholanoic acid) present in the human bile acid pool.

Unlike bile acid oxidation/epimerization, 7α-dehydroxylation appears to be restricted to free bile acids. The removal of glycine/taurine by BSHs is a prerequisite for 7α-dehydroxylation by intestinal bacteria[88]. The deconjugation and 7α-dehydroxylation of bile acids increase their pKa and hydrophobicity, allowing a certain degree of recovery by passive absorption across the colonic epithelium. However, their increased hydrophobicity is also associated with increased toxicity. High concentrations of secondary bile acids in feces, blood, and bile have been linked to the pathogenesis of cholesterol gallstone disease and colon cancer[89].

Enterohepatic circulation

The interactions of bile acids with the intestine, including ileal bile acid transport and its regulation, have been reviewed in a separate paper of this series[90]. Here we shall briefly comment on the major points of this aspect of bile acid physiology. Bile acid molecules are mostly confined to the territories of the so-called enterohepatic circulation, which includes the liver, the biliary tree, the intestine and the portal blood with which bile acids are returned to the liver. Upon completion of their digestive tasks, most intestinal bile acids (95%) are recovered by active transport in the intestine, mainly in the ileum. Active uptake of bile acids at the apical membrane of intestinal epithelial cells is performed by the apical sodium-dependent bile acid transporter (ASBT, gene symbol *SLC10A2*). This carrier is a symporter able to co-transport two sodium ions together with one molecule of bile acid[91]. For a long time, the efflux of bile acids from intestinal cells across the basal membrane has been a matter of controversy. The currently accepted concept is that this process is mainly accounted for by the heterodimeric organic solute transporter alpha and beta (OSTα-OSTβ)[92].

Albumin-bound bile acids that reach the liver mainly *via* the portal blood but also, although to a lesser extent, *via* the hepatic artery, are efficiently removed by transport proteins located at the sinusoidal membrane of hepatocytes. The first-pass extraction fraction ranges from 50% to 90%, depending on the bile acid structure[93]. The uptake of conjugated bile acids is largely sodium-dependent and is performed by the Na-taurocholate co-transport polypeptide (NTCP, *SLC10A1* gene)[94]. Sinusoidal sodium-independent bile acid uptake also occurs. This process is carried out by members of the family of organic anion transporting polypeptides (OATP), mainly the OATP1B1 and OATP1B3 isoforms[95]. In the overall process of bile acid transport from blood to bile, canalicular secretion is the limiting step. This transport for monoanionic amidated bile acids, which constitute the majority of secreted bile acids, is ATP-dependent and is mainly performed by the bile salt export pump (BSEP, gene symbol *ABCB11*)[96]. Highly hydrophobic bile acids, such as LCA, can be sulfated in human hepatocytes as a means of reducing its toxicity by increasing its water-solubility. Bile acids conjugated with sulfate or glucuronic acid are dianionic and are transported by other canalicular pumps, such as MRP2 (*ABCC2* gene)[97] and BCRP (*ABCG2* gene)[98].

The high specificity of these hepatic and intestinal carrier proteins for bile acids accounts for the low levels of these compounds in peripheral blood, commonly below 10 μmol/L in healthy subjects[99].

PATHOPHYSIOLOGY OF BILE ACIDS

Defects in bile acid synthesis

Defects in bile acid synthesis are uncommon genetic disorders that account for approximately 1%-2% of cholestatic disorders in children[100]. The inheritance of

Table 2 Inborn defects in bile acid synthesis and biotransformation

Impaired process	Defect localization	Consequences
Sterol ring modification	Cholesterol 7α-hydroxylase (CYP7A1)	Increased hepatic cholesterol. In adults, LDL hypercholesterolemia and cholesterol gallstones
	Oxysterol 7α-hydroxylase (CYP7B1)	Accumulation of monohydroxyl bile acid species with marked cholestatic and hepatotoxic capabilities. Severe neonatal liver disease
	3β-Hydroxy-C27-steroid dehydrogenase/somerase (HSD3B7)	Cholestatic jaundice and malabsorption of lipids and lipid-soluble vitamins
	δ-4-3-Oxosteroid 5β-reductase (AKR1D1)	Accumulation of δ-4-3-oxo- and allo(5α-H)-bile acids. Liver disease rapidly progressing to liver failure
Side-chain modification	27-Hydroxylase (CYP27A1)	Cerebrotendinous xanthomatosis
	25-Hydroxylase (CH25H)	Low levels of primary bile acids in serum and increased urinary excretion of typical bile alcohols
	α-Methylacyl-CoA racemase (AMACR)	High concentrations of (25R) trihydroxy-cholestanoic acid in urine, bile, and serum
	Complete or partial absence of peroxisomes	Zellweger syndrome Infantile Refsum disease Neonatal adrenoleukodystrophy Hyperpipecolic acidemia
	Altered peroxisomal enzymes	Pseudo-Zellweger syndrome Pseudo-neonatal adrenoleukodystrophy X-linked adrenoleukodystrophy
Bile acid amidation	Bile acid acyltransferase (BAAT)	Absence of taurine or glycine conjugates. Enhanced proportion of sulfate and glucuronide conjugates
	Bile acid-CoA ligase?	Absence of taurine or glycine conjugates. Enhanced proportion of sulfate and glucuronide conjugates

these defects is autosomal and recessive. The resulting liver diseases vary from mild to severe, depending on the particular alteration. The most common clinical presentation is progressive cholestasis of infancy, although other clinical manifestations, such as advanced liver disease at birth, neonatal hepatitis or the development of liver disease in later childhood, can also occur. When the enzymatic defect results in an accumulation of toxic monohydroxylated and/or unsaturated oxo-bile acids, many of which are cholestatic[101], the progression of liver disease is usually rapid. Recent evidence suggests that certain cholestatic liver diseases in adults may also be due to an inherited defect in bile acid biosynthesis[102].

Diagnosis is accomplished by analysis of the profile of bile acid species and their precursors and/or metabolites in body fluids, using laboratory techniques such as fast atom bombardment-mass spectroscopy and gas chromatography-mass spectroscopy. Early diagnosis is critical for these patients, because several of these disorders can be successfully treated with the dietary addition of bile acids. This has a dual purpose: first, to replace the essential primary bile acids absent, and second, to down-regulate bile acid synthesis by negative feedback inhibition, thus reducing the production of abnormal toxic intermediate metabolites by hepatocytes bearing the defect.

As will be commented below in detail, inborn errors affecting the enzymes involved both in the modification of the sterol nucleus and the side-chain, as well as in side-chain amidation, have been identified (Table 2). Moreover, the absence or impaired function of peroxisomes also results in alterations in bile acid metabolism that accompany the other signs characterizing each syndrome (Table 2).

Defects in the modification of the sterol nucleus

At least four inborn errors affecting enzymes that modify the sterol rings have been identified. Three of them are associated with progressive liver disease.

Defect in cholesterol 7α-hydroxylase: The defect in the key enzyme of the classical pathway of bile acid synthesis, cholesterol 7α-hydroxylase (CYP7A1), has been associated with a decrease in bile acid production *via* the classical pathway, which is compensated by activation of the alternative acidic pathway[103]. In these individuals, hepatic cholesterol contents are increased and, in adults, LDL hypercholesterolemia and cholesterol gallstones are commonly present. However, usually there is no evidence of liver disease.

Defect in oxysterol 7α-hydroxylase: A defect in the conversion of 27-hydroxy-cholesterol to 7α,27-dihydroxy-cholesterol due to a deficiency in oxysterol 7α-hydroxylase (CYP7B1), an enzyme specifically involved in the acidic pathway, causes severe neonatal liver disease. This is probably due in part to the accumulation of monohydroxyl bile acid species, with marked cholestatic and hepatotoxic capabilities[104]. This defect, resulting from a mutation in the gene, reveals the importance in humans of this alternative pathway in early life.

Defect in 3β-hydroxy-C27-steroid dehydrogenase/isomerase: This enzyme catalyzes the oxido-reduction of the 3β-hydroxyl group of 7α-hydroxycholesterol. Its deficiency is the most common defect in bile acid synthesis[105,106]. Individuals with autosomal recessive mutations in the encoding gene, *HSD3B7*, fail to

synthesize bile acids normally and develop a form of progressive liver disease characterized by cholestatic jaundice and malabsorption of lipids and lipid-soluble vitamins.

Defect in δ-4-3-oxosteroid 5β-reductase: The absence of this cytosolic enzyme results in a lack of the ability to reduce the double bond between C-4 and C-5 of the sterol A-ring, and thus to convert 3-oxo intermediates into the corresponding 3α-hydroxyl products, an essential step in major bile acid synthesis. This defect results in a markedly reduced primary bile acid synthesis and a concomitant accumulation of δ-4-3-oxo- and allo(5α-H)-bile acids[107]. A clinical presentation resembling that of neonatal hepatitis is typical, together with rapidly progressive liver disease and liver failure in infancy. Treatment with bile acid replacement therapy provides beneficial results.

Defects in the modification of the side-chain

Several inborn errors affecting single enzymes involved in the modification of the cholesterol side-chain to produce C_{24} bile acids have been identified. Additionally, because β-oxidation of the side-chain occurs in peroxisomes, peroxisomal disorders can also affect bile acid synthesis, accompanying other manifestations typical of each syndrome[108].

Defect in sterol 27-hydroxylase: A mitochondrial sterol 27-hydroxylase (CYP27A1) deficiency accounts for the development of so-called cerebrotendinous xanthomatosis (CTX)[109]. Regarding the biosynthesis of bile acids, this defect specifically interferes with the initial modifications of the cholesterol side-chain, resulting in downstream production of bile alcohols and a decreased synthesis of primary bile acids[110,111]. In general, CTX must be considered a progressive lipid storage disease characterized by diarrhea (the earliest clinical manifestation, affecting approximately 75% of affected infants), cataract (appearing in the first decade of life), tendon xanthomas (adolescent- to young adult-onset), and neurologic alterations, such as dementia, psychiatric disturbances, pyramidal and/or cerebellar signs, and seizures (adult-onset). Owing to the formation of deposits of cholesterol and cholestanol, xanthomas appear on the Achilles tendon, the extensor tendons of the elbow and hand, the patellar tendon, and the neck tendons, but also in the lung, bones, and central nervous system.

Defect in 25-hydroxylase: An inborn error in sterol 25-hydroxylase (CH25H), which is involved in the alternative pathway for bile acid side-chain synthesis, has been suggested to account for the bile acid profile that is found in some cases of neonatal hepatitis syndrome. This is characterized by the presence of low levels of normal primary bile acids in serum and increased urinary excretion of typical bile alcohols[112].

Defect in alpha methylacyl-CoA racemase: Alpha methylacyl-CoA racemase (AMACR) deficiency is a recently described defect in bile acid side-chain oxidation[113,114]. This peroxisomal enzyme catalyzes the conversion of (25R) trihydroxy-cholestanoic acid (THCA) to its 25S isomer, a step that is essential for the subsequent peroxisomal β-oxidation to primary bile acids to be initiated. High concentrations of (25R) THCA are found in the urine, bile and serum of these patients.

Peroxisomal defects: Disorders in peroxisomal biogenesis (absence or diminished numbers of peroxisomes) and specific enzymatic defects in peroxisome-based lipid oxidation include a group of diseases (Table 2) that present an important phenotypical overlap, with variability in the type of liver disease developed[115]. Altered serum bile acids in patients with peroxisomal disorders have been described[116]. The cerebro-hepato-renal syndrome of Zellweger is probably the condition in which hepatic function is most affected; atypical mono-, di- and tri- hydroxy C-27 bile acids with low amounts of primary bile acids are present in this disease[117,118].

Apart from AMACR, other peroxisomal enzymes involved in the beta-oxidation of the bile acid side-chain are branched-chain acyl-CoA oxidase, D-bifunctional protein and sterol carrier protein X (SCPx). Deficiencies in these enzymes, associated with abnormalities in bile acid synthesis, have also been reported[108].

Defects in bile acid amidation

Defective bile acid conjugation, which is characterized by a complete absence of glycine and taurine conjugates of bile acids in biological fluids and a predominance of unconjugated CA, with small proportions of sulfate and glucuronide conjugates, has been reported[119]. Fat-soluble vitamin deficiency is severe. The authors proposed a defect in bile acid-CoA ligase, because no CA-CoA derivatives were detected in any biological fluids, although no genetic analyses were performed in that study. Until now, alterations in *SLC27A5* gene encoding for VLCS or bile acid-CoA ligase have not been described in humans, therefore deficiency of this enzyme remains a hypothetical disorder. However, as mice with deleted *SLC27A5* do have the expected phenotype[120], the possibility of the existence of the corresponding metabolic disorder in humans can be expected.

More recently, a similar biochemical phenotype caused by a homozygous mutation in BAAT has been reported in Amish individuals with familial hypercholanemia, pruritus, and fat malabsorption[121].

Defects in bile acid transport

Progressive familial intrahepatic cholestasis (PFIC) type 1 (Byler disease), type 2 and type 3 are genetic disorders of bile secretion in which the fundamental abnormality is the direct or indirect defective hepatobilary transport of bile acids and/or phospholipids. Inborn errors of biliary canalicular transport systems will be the subject of a separate paper of this series and have been previously

reviewed by others[122,123].

Among these diseases, PFIC type 2 is due to primarily impaired bile acid transport. In these patients, high levels of serum bile acids, together with severe progressive liver disease, are found. PFIC type 2 is caused by a mutation in the bile salt export pump (BSEP, gene symbol *ABCB11*)[124,125], the main agent responsible for the ATP-dependent secretion of monoanionic bile acids across the canalicular membrane[96].

The less severe variant of PFIC type 2 is benign recurrent intrahepatic cholestasis (BRIC) type 2. This is a mild condition characterized by intermittent crises of cholestasis without permanent liver damage. BRIC type 2 is also caused by mutations in *ABCB11*[126].

Mutations in the *BSEP* gene have also been related to the aetiology of intrahepatic cholestasis of pregnancy[127,128].

BILE ACIDS IN PATHOLOGY

Bile acids as deleterious agents

Owing to their amphipathic characteristics, bile acids may behave as detergent molecules, which in many cases is the primary cause of bile acid-induced damage when they accumulate in the liver and other organs[129]. In the cholestatic condition known as PFIC type 3, a defect in MDR3 (gene symbol *ABCB4*) occurs. MDR3 is the flopase involved in the translocation of phospholipids, mainly phosphatidylcholine, from the inner to the outer leaflet of the canalicular membrane[130]. The presence in the biliary lumen of bile acids, whose detergent ability is not buffered by phosphatidylcholine, causes attack and disruption by solubilizing the lipidic components of the apical membranes in hepatocytes and biliary epithelial cells. As a side effect, this results in an increased release of gamma-glutamyltranspeptidase, whose serum levels are higher than normal.

Elevated intracellular concentrations of bile acids, such as those attained in cholestasis, have been related to oxidative stress[131] and apoptosis, both in adult and fetal liver[132]. Bile acids may induce apoptosis both by directly activating the Fas death receptor[133] and by inducing oxidative damage that causes mitochondrial dysfunction, which in turn may trigger apoptosis[134,135].

Finally, a relationship between bile acids and cell proliferation also exists. Some bile acid species have been shown to modulate DNA synthesis during liver regeneration after partial hepatectomy in rodents[136,137], and the regenerative process is dependent on bile acid signaling through the nuclear receptor FXR[138]. Teratogenic[139] and carcinogenic[140] effects of the more hydrophobic bile acids have been reported. Thus, a role of bile acids in the etiology of cancer at different sites - colon, esophagus, or even non-digestive tissues such as breast - has been suggested[141,142]. Moreover, it has recently been shown that mice lacking FXR spontaneously develop liver tumours[143,144].

Secondary alterations in bile acid homeostasis

The normal hepatic synthesis and enterohepatic circulation of bile acids are altered in some pathological conditions. This can indeed be expected in chronic liver diseases such as hepatitis or cirrhosis, which indirectly impair bile secretion, but this is also the case in other pathologies that do not directly affect hepatocyte secretory function, but in which changes in bile acid metabolism secondary to the primary disease have been described. This group of diseases includes cystic fibrosis[145] and diabetes mellitus[146].

CONCLUSION

From the results obtained over the past three decades, it is becoming evident that bile acids can no longer be considered as simple detergent compounds that are useful in digestive processes. The list of their physiological roles, as well as that of the pathological processes in which they are involved either as etiological agents, mediators of the pathogenic process, or simply affected by disease-induced changes in the liver or the intestinal handling of these steroids, is long and still not complete. Moreover, owing to their peculiar physical-chemical and biological characteristics, the huge potential usefulness of bile acids in the development of pharmaceutical approaches as well as their use as natural drugs or as the basis for the synthesis of novel semisynthetic drugs is encouraging many different groups worldwide to invest efforts in this direction. There is no doubt that many new concepts, pharmaceutical tools and pharmacological uses of bile acids and their derivatives will emerge in the near future.

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TOPIC HIGHLIGHT

Jose JG Marin, Professor, *Series Editor*

Excretion of biliary compounds during intrauterine life

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Abstract

In adults, the hepatobiliary system, together with the kidney, constitute the main routes for the elimination of several endogenous and xenobiotic compounds into bile and urine, respectively. However, during intrauterine life the biliary route of excretion for cholephilic compounds, such as bile acids and biliary pigments, is very poor. Although very early in pregnancy the fetal liver produces bile acids, bilirubin and biliverdin, these compounds cannot be efficiently eliminated by the fetal hepatobiliary system, owing to the immaturity of the excretory machinery in the fetal liver. Therefore, the potentially harmful accumulation of cholephilic compounds in the fetus is prevented by their elimination across the placenta. Owing to the presence of detoxifying enzymes and specific transport systems at different locations of the placental barrier, such as the endothelial cells of chorionic vessels and trophoblast cells, this organ plays an important role in the hepatobiliary-like function during intrauterine life. The relevance of this excretory function in normal fetal physiology is evident in situations where high concentrations of biliary compounds are accumulated in the mother. This may result in oxidative stress and apoptosis, mainly in the placenta and fetal liver, which might affect normal fetal development and challenge the fate of the pregnancy. The present article reviews current knowledge of the mechanisms underlying the hepatobiliary function of the fetal-placental unit and the repercussions of several pathological conditions on this tandem.

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Key words: Bile acids; Bilirubin; Foetus; Liver; Placenta

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INTRODUCTION

In the adult liver, most cholephilic organic anions are taken up from the portal blood by hepatocytes across the basolateral plasma membrane by sodium-dependent and -independent carriers (Figure 1). These are members of two groups of proteins: (1) the organic anion-transporting polypeptides family (OATP, gene symbol *SLCO*), whose isoforms OATP1B1, OATP1B3 and to a lesser extent OATP1A2[1], play a major role in the uptake of cholephilic compounds by human hepatocytes; (2) the Na^+-taurocholate-cotransporting polypeptide (NTCP, *SLC10A1*)[2]. Several members of the organic anion transporter (OAT) and organic cation transporter (OCT) family (gene symbol *SLC22A*) collaborate in the uptake of a large variety of organic molecules by the liver.

The secretion of cholephilic compounds into bile is accounted for by export pumps located at the canalicular plasma membrane. These are proteins belonging to the ATP-binding cassette (ABC) superfamily, which, in this region of the hepatocyte include the P-glycoprotein or multidrug resistance protein (MDR1; *ABCB1*), able to transport organic and inorganic cations[3]; the sister of P-glycoprotein or bile salt export pump (BSEP; *ABCB11*), which constitutes the main secretory system for bile acids[4]; the isoform 2 of the multidrug resistance-associated protein (MRP2; *ABCC2*), which exports conjugated forms of bilirubin, bile acids and xenobiotics[5,6], and the breast cancer resistance protein (BCRP; *ABCG2*), able to export sulfated steroids, which probably include bile acids[7].

In normally functioning healthy adult livers, at least as far as the excretion of cholephilic compounds into bile is concerned, the expression levels of MRP1 (*ABCC1*) and

MRP3 (*ABCC3*) at the basolateral membrane of hepatocytes is low[8,9]. However when the biliary excretory route is impaired, such as in cholestasis or endotoxemia, cholephilic compounds accumulate in hepatocytes, inducing an up-regulation of basolateral export pumps[10-12]. This acts as an adaptative response to reduce the cytotoxic effects of cholephilic compounds by pumping them back to the systemic circulation and accounts for an increased elimination of these substances into urine[13].

During pregnancy, owing to the immaturity of the fetal hepatobiliary excretory function, the existence of an alternative mechanism for the detoxification of cholephilic compounds produced by the fetus is required. The placenta, in collaboration with the maternal liver, carries out this function, which is very important for maintaining low bile acid and bilirubin levels in the fetal compartment. Moreover, the placenta also protects the fetal compartment, at least to a certain extent, from potentially toxic compounds coming from the maternal blood[14]. When the fetal-maternal homeostasis is altered, as happens during intrahepatic cholestasis of pregnancy, and these molecules accumulate in the *conceptus*, the consequences can be as serious as stillbirth and fetal death[15].

THE HEPATOBILIARY EXCRETORY FUNCTION DURING INTRAUTERINE LIFE

Fetal bile acid synthesis and maturation of the enzyme equipment required for bile acid and bile pigment metabolism precede the development of an efficient biliary-secretory system. Thus, although during intrauterine life bile acids are not required for digestive purposes, the fetal liver is able, from very early on during gestation, to synthesize primary bile acids, mainly cholic acid and chenodeoxycholic acid from cholesterol. Indeed, these two molecules are the major components of the human fetal bile acid pool[16-17]. The fetal bile acid pool is also characterized by the presence of molecular species with hydroxyl groups in positions that are unusual in bile acids found in adults. These are C-1, C-4 and C-6[18], which convert the molecule into a more hydrophilic one. This is believed to protect the fetal liver against the cytotoxic effect of less polar bile acid species when detoxification pathways are poorly developed. Another important characteristic of the fetal bile acid pool is the existence of bile acids with "flat" structures, accounted for by the presence of Δ4 or Δ5 insaturations or the alpha configuration of a hydroxyl in C-5[18]. Although the fetal gut is germ-free, the bile acid pool contains small amounts of secondary bile acids, such as deoxycholic acid and lithocholic acid, together with tertiary bile acids, such as ursodeoxycholic acid. This is probably due to placental transfer of these compounds from the maternal circulation[19].

Data collected from both rats[20] and humans[16,19,21] have revealed that serum bile acid concentrations are higher in fetuses than in their mothers, and that the composition of the bile acid species in both compartments is different. This has been explained in terms of the selective transplacental transfer of these cholephilic compounds[19], together with a different degree of maturation of the enzymatic machinery involved in bile acid metabolism[22]. The recently described role of bile acids as signaling molecules with several endocrine and paracrine functions[23] might account for the yet unknown physiological meaning of the early synthesis and special composition of the bile acid pool in fetuses.

From early gestation, the fetus also produces biliary pigments. The green pigment biliverdin, mainly the IXα isomer, is generated by cleavage of protoporphyrin IX by heme oxygenase[24,25] and is reduced to the golden pigment bilirubin IXα by the enzyme biliverdin reductase. The high production of bilirubin by the fetal liver, together with the still low activity in this organ of glucuronosyl transferase, the enzyme that produces more polar glucuronide conjugates to facilitate biliary excretion in adults, account for the higher concentrations of the unconjugated pigment in fetal serum than in maternal serum[19,26].

For many years, these bile pigments were considered mere waste products from heme metabolism, and the biological advantage of the conversion of the water-soluble and non-toxic compound biliverdin into the poorly water-soluble and neurotoxic compound bilirubin was not understood. Since the efficacy of biliverdin and bilirubin glucuronide transfer across the placenta is very poor, it was suggested that the formation of bilirubin from biliverdin may play a role in facilitating the elimination of heme-derived pigments in utero[27]. During the last decade, however, different studies have demonstrated the ability of bilirubin to protect cells against free radical damage both *in vitro* and *in vivo* in several tissues[24,28,29]. A recent work carried out by our group[30] revealed that, up to a certain degree of accumulation of bilirubin (below toxic levels), this pigment may help to protect the placental-fetal unit from maternal cholestasis-induced oxidative stress. Together with its direct antioxidant properties, bilirubin is also able to induce the expression of antioxidant systems. Thus, the current concept is that, when maintained in the physiological non-toxic range, bilirubin must be considered a beneficial compound[31].

In spite of the immaturity of fetal bile secretion, small amounts of bile acids have been detected in the gallbladder bile collected from human fetuses obtained from abortions older than 12 wk of age[32]. Regarding bile pigments, although the IXβ isomer of bilirubin constitutes only a small fraction of the total amount produced in the fetus[33], this more water-soluble isomer is the most abundant isomer found in fetal gallbladder bile and meconium[34,35]. The reason for this is two-fold: (1) bilirubin IXβ cannot easily cross the placenta; and (2) it can be excreted into bile without previous conjugation with glucuronic acid[36].

The fact that the expression of export pumps, such as Mrp2 and Bsep, only appears in rat fetal liver in the last third of gestation[37,38] is probably the cause of the low efficiency of this route of excretion during pregnancy. As previously commented, the serum levels of bile acids and bile pigments are higher in the fetus than in the mother. It is not known how cholephilic organic anions generated by the fetal liver reach the sinusoidal blood, but because some OATPs may act as bi-direction-

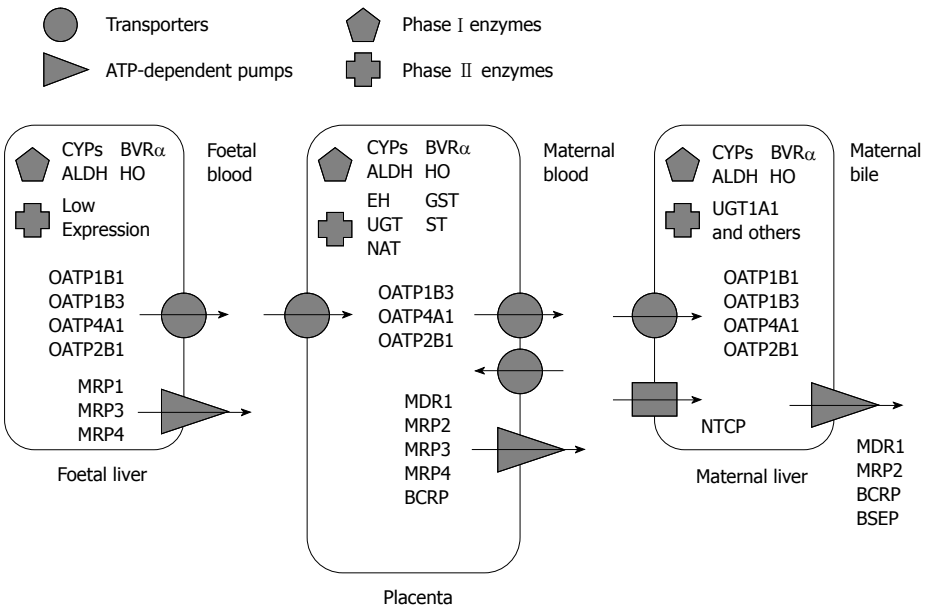

Figure 1 The foetal liver-placenta-maternal liver excretory pathway. Schematic representation of transporters (NTCP, sodium-taurocholate co-transporting polypeptide; OATP: Organic anion-transporting polypeptide), ATP-dependent pumps (BCRP: Breast cancer resistance protein; BSEP: Bile salt export pump; MDR: Multidrug resistance protein; MRP: Multidrug resistance-associated protein), and phase I (ALDH: Aldehyde dehydrogenase; BVR: Biliverdin reductase; CYP: Cytochrome P450 enzyme; HO: Heme oxygenase) and II (EH: Epoxide hydrolase; GST: Glutathione-S-transferase; NAT: N-acetyltransferase; ST: Sulfotransferase; UGT: UDP-glucuronosyl transferase) enzymes involved in excretion of biliary compounds during intrauterin life.

al transporters[39-41], they are good candidates for carrying out this function. Moreover, the expression of several OATP isoforms has been detected in rat fetal liver[38,42]. However, the abundance of these transporters in fetal liver is much lower than in adult liver, except for Oatp4a1[43] and its human orthologue OATP4A1[44]. Another possibility is that cholephilic compounds may exit the fetal liver *via* ATP-dependent pumps of the ABC family located in the basolateral plasma membrane. Supporting this concept is the higher expression of Mrp1 and Mrp3 in fetal than in maternal rat liver[38,42].

The accumulation of bile acids in fetal serum can have serious consequences, depending on the magnitude of the hypercholanemia[45]; in the most severe cases there is an increased risk of stillbirth and perinatal mortality[46], while in less severe conditions, maternal hypercholanemia can affect normal fetal development, the liver being one of the tissues most affected[47]. In fact, in a laboratory animal model of maternal hypercholanemia, the repercussions on fetal hepatobiliary function, although reversible, are maintained in young animals[20], and are characterized by a partial impairment in the ability of the liver to secrete organic anions, whereas the bile acid-induced biliary secretion of phospholipids, but not cholesterol, is increased[20,48].

The fetal kidney is able to secrete small amounts of organic anions into the amniotic fluid[17]. This, together with the detection of ABC proteins in the apical membrane of the yolk sac, has led to the suggestion that fetal membranes provide an additional route to protect the fetus against endogenous and xenobiotic compounds[49]. However, owing to the immaturity of the fetal renal system, the importance of this route in excreting cholephilic compounds during gestation is low[50-52].

ROLE OF THE PLACENTA IN THE EXCRETION OF BILIARY COMPOUNDS

Based on the foregoing it is clear that in contrast to what happens in the adult where the hepatobiliary system with the collaboration of the kidney are responsible for the biotransformation and elimination of bile acids, biliary pigments, drugs and food components, the main route for the elimination of these compounds during intrauterine life is their transfer to the mother across the placenta. Later on, the biotransformation and elimination into feces and urine is carried out by the maternal liver and, to a lesser extent, by the maternal kidney, respectively.

Excretion of bile acids

As mentioned above, there is a transplacental gradient for bile acids in the fetal-to-mother direction, except for secondary and tertiary bile acids, which are more abundant in maternal serum[19]. Several experimental lines of evidence suggest that simple diffusion is not the main mechanism by which these organic anions cross the human placenta[53]. In fact, ATP-dependent mechanisms account for the vectorial transfer of these compounds in the fetus-to-mother direction[54]. This has important implications, because in situations of maternal hypercholanemia there is only a moderate increase in bile acid concentrations in fetal serum[47].

The human placenta is of the haemochorial type, i.e. only the endothelium of chorionic vessels, the stroma of chorionic villi and the trophoblast layer separate the fetal and maternal blood. This means that in order to eliminate fetal metabolic by-products across the placenta, they must cross these three components of the placental barrier. Once in the maternal blood, most foetal bile acids are eliminated in bile by the maternal liver and excreted into feces. Regarding this task, the maternal kidney only contributes slightly to the excretion of sulphated and glucuronidated species[55].

For several years there has been functional evidence for a mediated transport of cholephilic organic anions at both poles of human and rat trophoblasts[56]. Functional studies carried out on isolated human trophoblast mem-

brane vesicles have suggested the presence of an anion exchanger transport system for the uptake of bile acids across the basal membrane of trophoblasts[53]. The trans-activation of this transport system with bicarbonate[57], the fact that substrate specificity is not restricted to bile acids[58] and the different affinities found for bile acid species depending on the number of hydroxyl groups and amidation[59] have led to the speculation that in the fetal-side membrane of the trophoblast there are proteins, probably belonging to the OATP family, that could be responsible for the uptake of organic anions by the trophoblast from fetal blood.

With respect to the opposite pole of the polarized epithelial trophoblastic cells, functional studies using plasma membrane vesicles have demonstrated that the transfer of bile acids toward the maternal circulation is dependent on ATP hydrolysis, in both human[54] and rat[60] placentas. However, it has been suggested that in the absence of ATP, bile acids could also cross this membrane by electrogenic-facilitated diffusion[61] and/or anion exchange[62]. The ATP-dependent system has higher substrate affinity, while the ATP-independent system has greater capacity[54]. These data suggest that in the apical membrane of trophoblasts, ABC proteins may be involved in pumping out bile acids towards the mother and, that proteins of the OATP family may participate in the ATP-independent component of this transport.

Concerning human OATPs, the mRNA of OATP1A2, OATP1B1 and OATP1B3 was detected in human placenta using real-time quantitative PCR[63,64]. The expression levels of OATP1A2 and OATP1B1 were shown to be very low at term, although they were detected at higher levels early during gestation[65]. Both OATP2B1 and OATP4A1 were also highly expressed in human placenta[44,66]. However, the former is not believed to be involved in the transport of bile acids[67], while the latter, which is considered to be a thyroid hormone carrier, is able to transport several bile acids[44].

Regarding the expression of Oatps in rat placenta, several isoforms have been detected. Under normal physiological circumstances, mRNA expression levels at term were low for Oatp1a1, Oatp1b4 and Oatp1b2, but high for Oatp4a1[42]. However, maternal cholestasis induces an up-regulation of these transporters, which is further enhanced when pregnant rats are treated with UDCA[68]. In addition, Oatp2b1 is also present in rat placenta and its ability to transport taurocholate has been described[69].

Little is known about the sub-tissue and sub-cellular localization of OATPs in the placenta. It has been suggested that OATP2B1 would be localized in the basal plasma membrane of the trophoblast[66], whereas OATP4A1 has been detected in the apical plasma membrane[44].

Regarding ABC proteins, several members of this superfamily are expressed in the placenta. The main system accounting for bile acid excretion into bile, BSEP, has been detected at very low levels in human and rat placentas at term[42,64,70], but at higher levels during the first trimester of pregnancy in humans[65].

Some members of the MRP family with the ability to transport biliary compounds - MRP1, MPR2, MRP3 and MRP4 - have been identified in human placenta[64,71]. The mRNA expression levels of MRP1 are higher in placenta than in liver; the abundance of MRP2 in the placenta is low compared to liver, and the expression levels of MRP3 and MRP4 are similar and low in both tissues, respectively[64].

The available data for the rat orthologues Mrp1, Mrp2, Mrp3 and Mrp4 suggest that they have similar expression patterns as those described for human isoforms[68]. Moreover, it has been observed that, at least in rats, there is a strong up-regulation of these transporters during maternal cholestasis, which can contribute to the protection of the fetus against the high concentrations of bile acids and biliary pigments existing on the maternal side of the placenta under these circumstances[68].

The cellular localization of some of these proteins in the placenta is controversial. MRP1, MRP2 and MRP3 have been detected by immunofluorescence and Western blotting in the apical membrane of the syncytiotrophoblast[71], and MRP1 is also expressed in fetal blood vessels[71], and in the basal membrane of the syncytiotrophoblast[72].

Another important member of the ABC family is the breast cancer resistance protein (BCRP, *ABCG2*), also known as ABC placental protein (ABCP) due to its high expression in this organ[73]. This protein is able to export a broad range of substrates, which have been reported to include bile acids[7]. BCRP has been detected in the apical membrane of trophoblasts and in fetal vessels[64,74]. Both the mRNA and protein levels of Bcrp are higher in rodent placenta during mid-gestation but decrease at term[75].

Excretion of biliary pigments

The endothelium of chorionic vessels and the syncytiotrophoblast are exposed to high concentrations of hemoglobin through their direct contact with fetal and maternal blood, respectively. Hemoglobin and free heme can undergo auto-oxidation to produce superoxide (O_2^-) and H_2O_2, which in turn promote the formation of other highly reactive and damaging radical species. These include lipid peroxides and the very reactive hydroxyl radical if trace amounts of free iron are available[76]. Heme can be degraded either enzymatically or chemically. Both mechanisms utilize molecular oxygen (O_2) and require a reducing agent. In the reaction catalyzed by heme oxygenase (HO), NADPH is the source of the reducing equivalent[77].

HO is a microsomal enzyme that induces the cleavage of heme, a pro-oxidant, to produce the biliary pigment biliverdin, iron and carbon monoxide (CO)[77]. There are three HO isoenzymes: HO-1 is a 32 kDa protein also known as heat-shock protein (HSP) 32, which is expressed at high levels in spleen and liver. HO-1 can be induced by several stimuli including hypoxia and hyperoxia[78,79]. The induction of HO-1 was coupled to the synthesis of the iron-sequestering protein, ferritin[80]. Ferritin avidly binds iron and interrupts the redox cycling of iron, thereby preventing iron from being useful as a catalyst for oxidant stress[81]. Subsequent studies

have demonstrated that the induction of HO-1 is also coupled to the synthesis of iron-exporting proteins and hence a critical role of HO-1 in maintaining iron homeostasis *in vivo* has been suggested[82].

HO-2 is a 36 kDa protein that is widely distributed in tissues throughout the body, where it is constitutively expressed and not readily inducible[83]. HO-2 appears to have an additional function as a "heme/oxygen cell sensor", accounted for by the presence of an oxygen-sensing consensus region in the sequence of the gene[84].

As compared with the other two isoforms, HO-3 has low catalytic activity[85]. The differences between the HO isoforms also include the control of their expression, which is probably due to differences in the regulatory elements present in their promoter regions[77].

The expression of HO in human placenta has been studied extensively. The contribution of this enzyme to normal placental function is based on its sub-tissue localization, its enzymatic activity and the ability of the HO-related by-products to exert physiological effects on placental and fetal tissues[86]. Using RT-PCR, the amounts of mRNA encoding both the HO-1 and HO-2 isoforms have been measured in placental tissue. These studies demonstrated an elevated expression of HO-2 as compared to that of HO-1[87-89]. Moreover, the placental expression of both HO-1 and HO-2 increased as gestation advanced[88].

The cytoprotective properties of HO are partly due to the products of its activity, such as CO and biliary pigments. Notably, while the clinical toxicity of CO is clearly recognized, much smaller quantities of CO are remarkably cytoprotective, antiapoptotic, vasorelaxant, and anti-inflammatory[90]. As has already been mentioned, biliary pigments, long regarded to have adverse consequences in hyperbilirubinemic states, are now recognized as anti-inflammatory and antioxidant when present in low concentrations.

Biliverdin-IXα and subsequently bilirubin-IXα are the major biliary pigments in humans. However, small amounts of the other three isomers are also generated depending on the position of the protoporphyrin IX that is cleaved. These include biliverdin-IXγ, biliverdin-IXδ and biliverdin-IXβ, which is the most abundant of these three pigments in humans and other mammals[35].

Biliverdin, which is produced by HO-1 and HO-2 activity, is further biotransformed to bilirubin by biliverdin reductase: mainly biliverdin-IXα reductase (BVRα). This enzyme also functions as a kinase and as a transcription factor in the MAPK signalling cascade[91]. BVRα is expressed in many tissues[92], including the placenta[27]. Bilirubin is conjugated in the liver with glucuronic acid by bilirubin uridine diphosphate-glucuronosyl transferase-1A1 (UGT1A1)[93] prior to being secreted into bile. Owing to the immaturity of the fetal liver, no hepatobiliary elimination of bilirubin occurs, at least at a physiologically relevant rate.

Unconjugated bilirubin concentrations are higher in fetal than in maternal serum[19,26]. Several factors contribute to the existence of this gradient. In the fetus, there is a very active heme catabolism, and hence a high rate of bilirubin production, together with a very low expression of bilirubin uridine diphosphate-glucuronosyl transferase in the liver[94]. Moreover, simple diffusion is not the major route for the placental transfer of biliary pigments[56].

In the presence of reactive oxygen species, bilirubin is oxidized to biliverdin and then converted back into bilirubin by BVRα[95]. Thus the biliverdin-bilirubin tandem acts as an efficient scavenger of reactive oxygen species and inhibits lipid oxidation both *in vitro* and *in vivo*[96,97]. Bilirubin is also an effective antioxidant of peroxynitrite-mediated protein oxidation and inhibits the production of superoxide by blocking the activation of NADPH oxidase[98,99]. Sub-toxic bilirubin concentrations have direct anti-oxidant properties and indirect beneficial effects against cholestasis-induced toxicity during pregnancy, such as the enhanced expression of several elements of the anti-oxidant defence system, i.e. BVRα, SVCT1 and SVCT2, as well as several nuclear receptors sensitive to activation by biliary compounds[100]. This function is mainly dependent on the expression of BVRα, which has been found to be moderately up-regulated in the maternal liver-placenta-fetal liver trio in pregnant rats with surgically induced obstructive cholestasis during the last week of gestation[100]. However, beneficial antioxidant properties are limited to low bilirubin concentrations because at higher levels this pigment can also cause irreversible damage or even death when it is accumulated in the nervous system[101].

It has been suggested that the reduction of biliverdin to bilirubin could have the evolutionary advantage of facilitating the placental excretion of bile pigments by simple diffusion[102]. However, *in vitro*[103] and *in vivo*[104] studies have suggested that under normal physiological circumstances the major pathway for bilirubin placental transfer involves carrier-mediated transport across both poles of the plasma membrane of the human trophoblast[103]. Moreover, at least in rodents, bilirubin does not undergo any major biotransformation during its residence in the placenta[104]. The existence of vectorial properties for transplacental bilirubin transfer are consistent with the moderate increases in serum bilirubin concentrations observed in the fetuses of pregnant rats with marked hyperbilirubinemia due to common bile duct ligation[47]. The mechanism for the placental uptake of fetal biliary pigments is not completely understood. Proteins of the OATP family, in particular human OATP1B1 and OATP1B3, have been reported to confer the ability to take up unconjugated bilirubin when expressed in *Xenopus laevis* oocytes[63]. However, the mRNA of OATP1B1 is almost absent in isolated human trophoblast cells, whereas OATP1B3 is clearly expressed in this epithelium, although at low levels[63].

Inside trophoblast cells, bilirubin is probably partly bound to lipids and proteins such as glutathione-S-transferase[105]. Functional studies have suggested that bilirubin might be exported across the apical pole of the trophoblast *via* an ATP-dependent mechanism[103]. Whether one or several isoforms of MRPs expressed in human[71] and rat[42,68] placenta are involved in this process is not known.

MRP2, and probably MRP1, are also able to perform ATP-dependent transport of bilirubin glucuronides[5]. However, owing to the low UDP-glucuronosyl transferase activity of the fetal liver and the absence of placental biotransformation of unconjugated bilirubin during transplacental transfer[106], MRP2 and MRP1 are not expected to play an important role in bilirubin transfer across the placenta.

It has been shown that biliverdin itself is poorly transferred-without prior reduction to bilirubin - across the guinea pig[26] and rat[106] placenta. However, biliverdin is able to inhibit bilirubin transfer in rat placenta when co-administered through the umbilical artery of *in situ* perfused rat placentas[106]. The transport of biliverdin from the trophoblast toward the mother is very poor and/or that placental biotransformation of biliverdin into bilirubin is very efficient. Part of the endogenous biliverdin produced by the fetus could be transformed into bilirubin by the fetal liver prior to being taken up by the placenta, because the expression of BVRα in fetal liver is even higher than in rat placenta[100].

Among the transporters involved in fetal biliverdin uptake by rat placenta, several OATPs, in particular Oatp1a1, may be involved[106]. Once in the placenta, and prior to being transferred to the mother, biliverdin is extensively converted into bilirubin by BVRα, which is highly expressed in this organ[106]. The small amount of biliverdin that reaches the maternal blood is efficiently taken up, probably in part by Oatp1a1, Oatp1a4 and Oatp1b2, and biotransformed into bilirubin, which joins the fetal bilirubin transferred by the placenta, to be eliminated mainly through secretion into the bile by the maternal liver[106].

PROTECTION AGAINST DRUGS AND TOXINS

Fetal exposure to foreign molecules is partly dependent on the maternal capacity to eliminate such compounds and on the ability of the xenobiotics to cross the placenta. One important characteristic of the placenta is that this organ undergoes continuous development. This implies the existence of changes that must be compatible with the maintenance of a partially permeable epithelial barrier required to provide protection against exposure to potentially harmful substances present in the maternal blood[56,107,108]. Therefore, before any pharmacological interventions, the different stages of pregnancy should be considered, because these will determine both the permeability of the placental barrier and the vulnerability of the conceptus to xenobiotics[109].

Although most drugs administered during pregnancy may cross the placenta to some extent, the magnitude of this depends on the size and structure of the molecule. Diffusional transfer across the placenta for drugs with a molecular weight higher than 500 Da is usually very restricted[110]. Liposolubility and ionization are strong determinants for drug diffusion across the placenta. For instance, several penicillins, in spite of being strong acids, can be efficiently transferred across the human placenta, probably by simple diffusion[111]. Among weak-base drugs, acetaminophen, phenobarbital, phenytoin and clonidine are able to cross the placenta at a high rate, probably by simple diffusion[112]. Nucleoside analogue reverse transcriptase inhibitors (NRTIs) are molecules with low molecular weight and low protein binding, so most of them are also able to cross the placenta by simple diffusion and are concentrated in the amniotic fluid[113].

Carrier-mediated uptake

Although most transporters localized at the plasma membrane of cells and forming part of the placental barrier have specific physiological substrates, some of them also transport structurally similar compounds. In some cases, however, there are no known physiological substrates and only certain xenobiotics have been reported to be transported by them. Moreover, some of the xenobiotics able to cross the placental barrier may have the ability to affect gene expression. This may result in a decrease in the expression of placental transporters, which may affect their ability to accomplish their physiological roles and eventually lead to an enhanced entry of drugs into placental tissue[114].

The placenta expresses some isoforms of monocarboxylate transporters (MCTs)[115]. The primary substrate of MCTs in placenta is lactate, although pyruvate and β-hydroxybutyrate are also transported. Placental MCTs exert a significant influence on the transfer across the maternal-fetal interface of drugs such as valproate, benzoate, salicylates, statins, nateglinide, and foscarnet[114,115].

Equilibrative nucleoside transporters (ENTs) are widely distributed and have broad substrate specificity. There is evidence of the presence of two ENT isoforms in the human placenta: ENT1 (*SLC29A1*) and ENT2 (*SLC29A2*)[116,117]. Moreover, concentrative nucleoside transporters (CNTs), CNT2 (*SLC28A2*) and CNT3 (*SLC28A3*) are also expressed in human placenta. Both ENT1 and ENT2 are able to transport a wide variety of therapeutic agents such as the anticancer drugs cytarabine and gemcitabine and the antiviral drugs zalcitabine (ddC) and zidovudine[117,118], and they therefore probably play a role in fetal exposure to these types of drugs.

Regarding amino acids and monoamines, 17 mammalian transport systems for amino acids have been functionally identified in the human placenta[119,120]. The interaction of xenobiotics with amino acid transport systems in the syncytiotrophoblast may result in a deficit in the transport of amino acids across the placenta. This seems to be the case for cocaine, which readily crosses the placental barrier and enters the fetal circulation. This constitutes a potential cause of adverse effects on the developing fetus in pregnant women consuming this drug[121]. Maternal smoking during pregnancy also decreases the ability of the placenta to efficiently take up amino acids and hence affects the overall transfer of these important metabolites from the maternal to the fetal circulation[121].

Additionally, cocaine may also interact with other placental carriers, such as those involved in monoamine

transport[121]. This may affect serotonin and noradrenaline transport across the apical (maternal-facing) plasma membrane of the trophoblast[122]. Moreover, antidepressants (fluoxetine, paroxetine, sertraline, citalopram, and desipramine) as well as cocaine are inhibitors of monoamine transporters but are not transportable substrates. In contrast, amphetamines are transportable substrates for monoamine transporters, thereby gaining access into the placenta and fetus[122,123].

Many xenobiotics are substrates of OATP isoforms[124], which are expressed in the human placenta[63,66]. These transporters have partially different and overlapping substrate preferences for a wide range of exogenous organic solutes, including gadodexate, ouabain, iloprost, Gd-B 20790, methotrexate, rifampicin, the endothelin receptor antagonist BQ-123, the thrombin inhibitor CRC-220, the opioid receptor agonists D-penicillamine-(2,5)-enkephalin (DPDPE) and deltorphin Ⅱ, the angiotensin-converting enzyme inhibitors enalapril and temocaprilat, the HMG-CoA reductase inhibitor pravastatin, and the antihistamine fexofenadine, in addition to several cytostatic derivatives obtained by coupling bile acid moieties to chlorambucil or cisplatin[125-127]. Some OATP isoforms have also been shown to transport bulky organic cations[124,128]. This suggests that isoforms detected in placenta could serve as a route for the transfer of anions and relatively hydrophilic cationic organic drugs.

Organic cations can be transferred across the placenta using a different route. At least one member of the subfamily of carriers for organic cations (OCTs), namely OCT3, is very abundantly expressed in the human placenta[129,130]. Examples of OCT3 substrates include cimetidine, MPP^+, agmatine, tetraethylammonium, and prazosin[131]. The sodium-dependent carnitine transporter (OCTN2) also belongs to the *SLC22A* family and is expressed in human placenta[132]. OCTN2 transports a variety of organic cations including tetraethylammonium, nicotine, MPP^+, pyrilamine, cimetidine, clonidine, procainamide, quinidine, quinine, and verapamil[133] and certain β-lactam antibiotics of zwitterionic nature[134].

Metabolic barrier

During the first trimester of pregnancy, a broader variety of xenobiotic-metabolizing enzymes are expressed in the placenta as compared to at term[135,136]. However, placental expression of phase I and Ⅱ metabolizing enzymes is moderate and probably more closely involved in the endocrine functions of this organ than in the metabolism of xenobiotics[135]. The placenta expresses several cytochrome P450 enzymes (CYPs) at mRNA levels that increase throughout pregnancy. Although placental CYPs are capable of metabolizing several xenobiotic compounds at term[135,136], only a few of these enzymes are actually functionally active[137,138]. Moreover, the abundance of some of these CYPs has been shown to be affected by exposure to xenobiotics, as occurs in tobacco-smoking pregnant women[138,139]. Other phase I metabolizing enzymes such as aldehyde dehydrogenases (ALDHs) participate in the detoxification of endogenous and exogenous compounds, including ethanol. The presence of this activity in human placenta may be relevant in the toxicity of a number of substances and for the gestational consequences of alcohol consumption[140].

Among phase Ⅱ enzymes, glutathione-S-transferases, epoxide hydrolase, N-acetyltransferases, sulfotransferases, and UDP-glucuronosyl transferases are expressed at moderate levels in the placenta and have been shown to be involved in the detoxification of several xenobiotics[141]. In contrast, drug- and toxin-induced upregulation of biotransforming enzymes can lead to an enhanced production of reactive metabolites able to interact with DNA, resulting in the formation of DNA adducts[142]. This may challenge the normal development of the *conceptus*. Indeed the levels of smoking-related adducts in the placenta have been inversely correlated with offspring birth weight[143].

Export systems

ATP-dependent efflux transporters expressed in the apical membrane of placental syncytiotrophoblasts are very important in limiting the magnitude of drug penetration across the placental barrier, hence reducing fetal drug exposure. The superfamily of ABC proteins includes a large number of members with the ability to translocate a broad variety of substrates across extra- and intracellular membranes. These proteins are involved in many physiological processes, such as sterol homeostasis, immune mechanisms, and the transport of endogenous and xenobiotic substances such as sugars, amino acids, metal ions, peptides and proteins, and a large number of hydrophobic compounds and metabolites. Several members of three families of ABC transporters, ABCB, ABCC and ABCG, known to be involved in multidrug resistance are major candidates for involvement in the placental barrier for drugs[144,145].

The first ABC transporter recognized to play a significant role in the placental barrier was MDR1[145]. MDR1 is abundantly expressed during pregnancy, and in particular in the syncytiotrophoblast[146]. The substrates of MDR1 are usually organic molecules ranging in size from about 200 Da to almost 1900 Da. Most of them are uncharged or weakly basic in nature, but some acidic compounds can also be transported. As a consequence, a large number of drugs from several pharmacotherapeutic groups are recognized as MDR1 substrates. Thus, placental MDR1 may contribute to the protection of the foetus from a wide variety of drugs, including antivirals and anticancer agents[147].

Other major efflux transporters involved in the protection of the developing fetus from exposure to these drugs are members of the MRP subfamily, involved in the transport of conjugates of several drugs and endogenous compounds, have been found in the human placenta. MRP2 is expressed in the syncytiotrophoblast, whereas MRP1 and MRP3 are expressed both in blood vessel endothelia and in the syncytiotrophoblast[71], and MRP5 is expressed in the basal membrane of syncytiotrophoblasts and around fetal vessels[148], where aside from its potential role in drug disposition this transport-

er may mediate the cellular efflux of 3',5'-cyclic nucleotides, cAMP, and cGMP, thus playing an important role in paracrine signal transduction.

BCRP expression in the placenta is possibly tightly controlled during pregnancy by pregnancy-related steroid hormones, growth factors, and cytokines[149]. BCRP transports a broad variety of conjugated or non-conjugated organic anions, but from a physiological point of view it is probably involved in the elimination of endogenous sulphate conjugates[150]. Substantial variations in BCRP expression have been observed in human placenta[151], suggesting that considerable variability could exist in the ability of the placenta to protect the fetus from exposure to drugs, xenobiotics and metabolites. Such variable expression and/or activity has been suggested to be due to genetic polymorphisms in the *BCRP* gene[151].

CONCLUSION

The immaturity of the fetal hepatobiliary system precludes the use of this mechanism of defence against endogenous and xenobiotic compounds during intrauterine life. Consequently, this function is carried out by a complex and efficient combined action of the placenta and the maternal liver. However, when one of these two members of the defensive tandem is impaired the overall function may be compromised, resulting in deleterious effects in the fetus. A better understanding of the molecular mechanisms involved in hepatobiliary excretory function during intrauterine life is needed to recognize the danger the fetus may face, to develop novel pharmacological tools to manipulate the placental transfer of xenobiotics, and to generate new drugs with enhanced or reduced ability to cross the placental barrier.

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Imaging features of solid pseudopapillary tumor of the pancreas on multi-detector row computed tomography

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Abstract

AIM: To retrospectively analyze the imaging features of solid-pseudopapillary tumors (SPTs) of the pancreas on multi-detector row computed tomography (MDCT) and define the imaging findings suggestive of malignant potential.

METHODS: A total of 24 consecutive cases with surgically and pathologically confirmed SPTs of the pancreas underwent preoperative abdominal MDCT studies in our hospital. All axial CT images, CT angiographic images, and coronally and sagittally reformed images were obtained. The images were retrospectively reviewed at interactive picture archiving and communication system workstations.

RESULTS: Of the 24 cases of SPTs, 11 cases (45.8%) occurred in the pancreatic head and seven (29.1%) in the tail. Eighteen were pathologically diagnosed as benign and six as malignant. MDCT diagnosis of SPTs was well correlated with the surgical and pathological results (Kappa = 0.6, $P < 0.05$). The size of SPTs ranged from 3 to 15 cm (mean, 5.8 cm). When the size of the tumor was greater than 6 cm (including 6 cm), the possibilities of vascular (8 *vs* 1) and capsular invasion (9 *vs* 0) increased significantly ($P < 0.05$). Two pathologically benign cases with vascular invasion and disrupted capsule on MDCT presented with local recurrence and hepatic metastases during follow-up about 1 year after the resection of the primary tumors.

CONCLUSION: Vascular and capsular invasion with superimposed spread into the adjacent pancreatic parenchyma and nearby structures in SPTs of the pancreas can be accurately revealed by MDCT preoperatively. These imaging findings are predictive of the malignant potential associated with the aggressive behavior of the tumor, even in the pathologically benign cases.

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Key words: Solid pseudopapillary tumor; Pancreas; Multi-detector row computed tomography; Malignant potential; Aggressive behaviors

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INTRODUCTION

Solid pseudopapillary tumors (SPTs) of the pancreas are rare and predominantly occur in young women in the second and third decades of life, with only about 8.3% of all cases reported in males[1,2]. SPT is usually a benign tumor with low-grade malignant potential; however, about 14.7% of cases demonstrate malignant behavior with recurrence and metastasis[3]. In contrast to the ductal adenocarcinoma of the pancreas, complete resection of the tumor could provide a more than 90% cure rate[4,5].

Currently, the imaging modalities such as computed tomography (CT) including multi-detector row computed tomography (MDCT), magnetic resonance

imaging (MRI), and ultrasonography (US) have been used for detection and characterization of pancreatic tumors in clinical practice[6,7]. It is well known that MDCT can provide more accurate delineation of normal and abnormal pancreatic morphology[6]. Furthermore, MDCT can be performed intrinsically for CT angiography (CTA) scanning, and the CTA images can be conveniently obtained by post-processing techniques at the dedicated workstation. However, to the best of our knowledge, there has been no published literature regarding the imaging features implying the malignant potential of the SPTs of the pancreas on MDCT.

This study aimed to retrospectively analyze the imaging features of SPTs of the pancreas on MDCT, and define the imaging findings suggestive of the malignant potential associated with aggressive behavior.

MATERIALS AND METHODS

Patient population

From June 2001 to June 2008, a total of 24 consecutive patients with surgically and pathologically confirmed SPTs of the pancreas, ranging in age from 11 to 64 years (mean 34.27 years; 21 females, three males), underwent preoperative abdominal MDCT studies in our hospital. Abdominal pain or discomfort was the major indication to undergo the imaging studies (87.5%; 21/24). The youngest female patient (11 years old) and a 21-year-old woman both presented with a big mass in the upper abdomen, while the remaining one demonstrated jaundice and elevation of liver enzymes, suggestive of impairment of liver function. The patients have been followed up for 3 mo to 7 years. Institutional review board approval and waiver of informed consent for this retrospective study were obtained.

Imaging techniques

All abdominal MDCTs were performed on a 4-slice or 16-slice multi-detector row CT scanner (LightSpeed QX/I or Lightspeed 16; GE Medical Systems, Milwaukee, WI, USA). All axial CT images were obtained during breath-holding before (non-enhanced), 25 s (arterial phase), 60 s (portal phase), and 90 s (hepatic parenchyma phase) after the initial administration of contrast materials. Contrast-enhanced MDCT was performed after the intravenous injection of iohexol (Omnipaque 300; Amersham, Shanghai, China) at a dose of 2 mL/kg body weight through an antecubital vein using a power injector (LF CT 9000; Liebel-Flarsheim, Cincinnati, OH) at a flow rate of 2.5 to 3.0 mL/s. The major scanning parameters were as follows: beam pitch, 1.5; beam collimation, 4-2.5 mm; gantry rotation time, 0.5-0.8 s; section thickness, 3.75 mm; reconstruction interval, 3.75 mm; and table speed, 15.0 mm/rotation. Axial images were reconstructed with a soft tissue algorithm. The CTA and reformed images were obtained using maximum intensity projection (MIP) or multiplanar volume reformation (MPVR) technique on the workstation. Besides MDCT, 15 cases underwent MRI, and 18 cases underwent US.

Imaging analysis

All images were retrospectively reviewed at interactive picture archiving and communicating system (PACS) workstations by two experienced abdominal radiologists (Wang DB and Chai WM) in consensus. Disputes in readings were resolved through consultation with a third experienced abdominal radiologist (Chen KM). The items included in the imaging analysis were: (1) size, location, and density, as well as the hemodynamics of the tumor; (2) vascular or capsular invasion, invasion of tumor into the adjacent pancreatic parenchyma, and involvement of nearby vessels as well as metastases in lymph nodes and other places in the upper abdomen. All the reviewers were unaware of the pathological nature regarding the benignity and malignancy for each case. Based on the imaging findings of the SPTs of the pancreas on MDCT, each case was given a diagnosis of either benign or malignant SPT.

Pathological studies

Histopathological and immunohistochemical studies were performed in all the cases. The diagnosis of SPTs by MDCT was compared with the pathological results.

Statistical analysis

The Fisher's exact test and Kappa test were introduced for comparison of imaging diagnosis and pathological results. A P value less than 0.05 was considered to indicate statistical significance. All statistical analyses were performed using the SPSS computer software (Version 13.0, Chicago, IL, USA).

RESULTS

Surgical and pathological results

Of the 24 cases, 18 were diagnosed as benign SPTs while the other six as malignant on pathological study. The size of SPTs ranged from 3 to 15 cm (mean 5.8 cm). One of the malignant SPTs metastasized to the liver unifocally and the regional lymph nodes. The metastatic lesions were also resected simultaneously during the operation of the primary tumor in the pancreatic head. There were another two cases of malignant SPT with regional lymph node metastasis found in surgery. The involvement of nearby vessels (n = 8, including two benign cases), adjacent organs including spleen (n = 1), duodenum (n = 1), and stomach (n = 1), and abutting pancreatic parenchyma (n = 6), as well as the peripancreatic fat (n = 6), was revealed during the operations (Table 1). One of them had a thrombus in the portal vein. Complete resection was performed for each case in this group.

Imaging features of SPTs on MDCT compared with pathological results

Table 1 summarizes the imaging features of SPTs on MDCT in comparison with pathological outcome. Of the 24 cases of SPTs, 11 (45.8%) occurred in the pancreatic head and seven (29.1%) in the tail (Table 1). The calcifications were identified in five cases (three

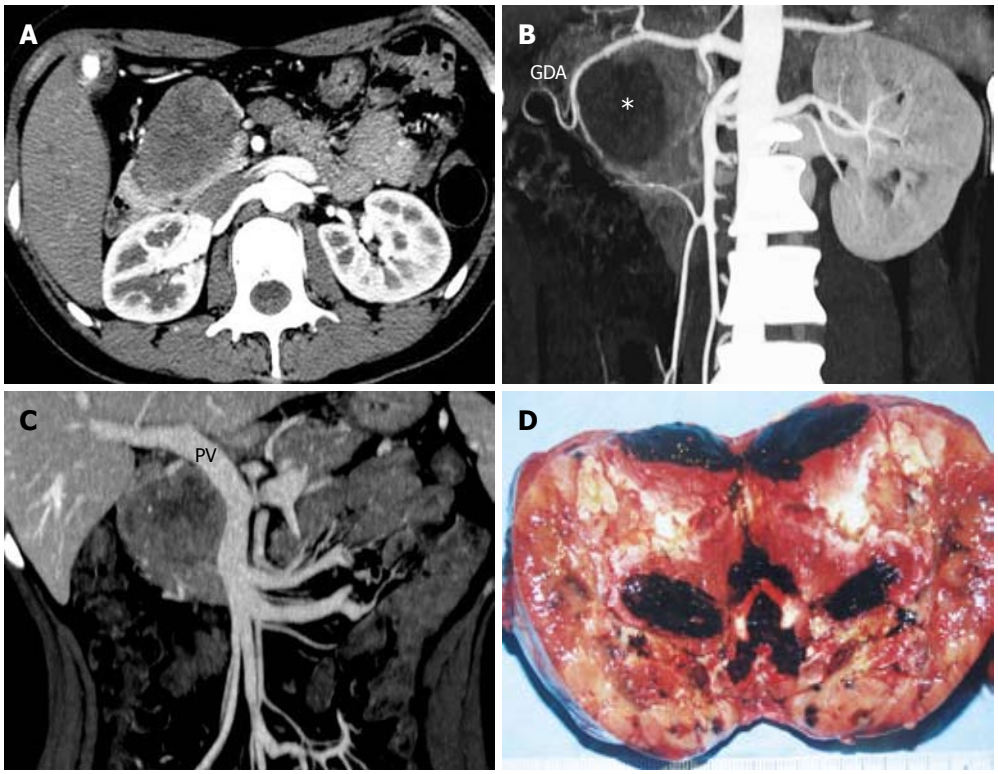

Figure 1 Pathologically confirmed benign SPT in a 24-year-old woman with abdominal discomfort for 1 year. A: Axial image at arterial phase revealed a low-attenuation mass in the pancreatic head. The surrounding vessels were displaced; B: The MIP CTA image identified that the tumor (*) displaced the gastroduodenal artery (GDA) without infiltration; C: The MPVR image demonstrated that tumor compressed the portal vein (PV) with a smooth border. There was no evidence suggesting invasion; D: Hemorrhagic and cystic areas (dark areas) were detected in the gross specimen. The capsule was intact.

benign, two malignant). On MDCT, the tumors were divided into three types according to the density: cystic, solid, and solid-cystic (mixed) (Figure 1). The mixed type comprised 58.3% (14/24) of the cases of SPT. Six malignant and three benign cases were identified with a disrupted capsule, whereas the other cases had an intact and smooth capsule. In this group, 50% (12/24) of the SPTs demonstrated moderate enhancement of the tumors after administration of contrast agents. Nine of the 12 cases were benign. However, among the six cases with marked enhancement, three were malignant. Peripheral and persistent enhancement in solid components occurred in 95.8% (23/24) of the cases. The MDCT identified all surgically and pathologically confirmed tumoral invasion into the adjacent structures and organs (spleen, duodenum and stomach) and metastases (lymph nodes, $n = 3$; liver metastasis, $n = 1$) in six malignant cases (Table 1). However, the adjacent pancreatic invasion ($n = 3$), pancreatic duct dilatation ($n = 1$), peripancreatic fat invasion ($n = 1$), and nearby vascular involvement ($n = 2$) in benign cases were also depicted on MDCT. The subsequent pancreaticoduodenectomy operation was performed on four of these cases, and pathological study revealed the benign nature in spite of encasement of the nearby portal vein. Therefore, eight cases were diagnosed as malignant SPTs by MDCT, including six categorized as malignant by pathology (75%; 6/8). Finally, MDCT diagnosis of SPTs was well correlated with the surgical and pathological results (Kappa = 0.6, $P < 0.05$) (Table 2).

When the size of the tumor was $\geqslant$ 6 cm, the possibilities of vascular involvement (8 *vs* 1) and capsular invasion (9 *vs* 0) increased significantly ($P < 0.05$) (Tables 1 and 3). The disrupted capsule detected on MDCT was attributed to the tumoral invasion into adjacent pancreatic parenchyma or peripancreatic structures by pathologically confirmed SPT in this group (Figure 2). Table 4 demonstrates that the portal vein, superior mesenteric vein, and splenic vein were vulnerable to being involved. Two pathologically benign cases with vascular involvement and disrupted capsule on MDCT presented with local recurrence and hepatic metastases during follow-up, about 1 year after resection of the primary tumors (Figure 3).

DISCUSSION

SPT of the pancreas is rare, mainly occurring in young women. It was first described by Frantz in 1959[8] and the tumor was named the Frantz tumor after the author. Over time, it has been designated with various names such as: solid and papillary tumor[9]; papillary cystic tumor[10]; solid-cystic tumor[11]; and solid and papillary epithelial neoplasm[12]. However, these names do not exactly reflect what is present either at the microscopic or macroscopic level. WHO in 1996 proposed the name 'solid-pseudopapillary tumor', which can depict two major histological features in the tumor: solid and papillary components[13]. In fact, SPTs have been subdivided by the WHO classification into: solid-

Table 1 Imaging features of SPTs on MDCT in comparison with surgical and pathological results *n* (%)

Imaging features on MDCT	Surgical and pathological results	
	Benign (*n* = 18)	Malignant or potentially malignant (*n* = 6)
Location		
Head	8 (33.3)	3 (12.5)
Neck	1 (4.2)	0
Body	2 (8.3)	1 (4.2)
Tail	5 (20.8)	2 (8.3)
Body-neck	1 (4.2)	0
Body-tail	1 (4.2)	0
Size		
< 6 cm	10 (41.7)	1 (4.2)
⩾ 6 cm	8 (33.3)	5 (20.8)
Density		
Cystic	5 (20.8)	0
Solid	3 (12.5)	2 (8.3)
Solid-cystic	10 (41.7)	4 (16.6)
With calcifications	3 (12.5)	2 (8.3)
Capsule		
Intact	15 (62.5)	0
Un-intact	3 (12.5)	6 (25)
Enhancement		
Mild	6 (25)	0
Moderate	9 (37.5)	3 (12.5)
Marked	3 (12.5)	3 (12.5)
Enhancing pattern and hemodynamics		
Peripheral and persistent enhancement	17 (70.8)	6 (25)
Central and persistent enhancement	1 (4.2)	0
Pancreatic or peripancreatic invasion		
Adjacent pancreatic invasion	3 (12.5)	6 (25)
Pancreatic duct dilatation	1 (4.2)	1 (4.2)
Peripancreatic fat invasion	1 (4.2)	6 (25)
Nearby vessels involvement	2 (8.4)	6 (25)
Direct invasion into adjacent organs	0	3 (12.5)
Regional lymph nodes metastasis	0	3 (12.5)
Liver metastasis	0	1 (4.2)

Table 2 Comparison of MDCT diagnosis and pathologic results in SPTs

MDCT	Pathologic results		Total
	Malignant or malignant potential	Benign	
Malignant	5	3	8
Benign	1	15	16
Total	6	18	24

Kappa = 0.6, $P < 0.05$.

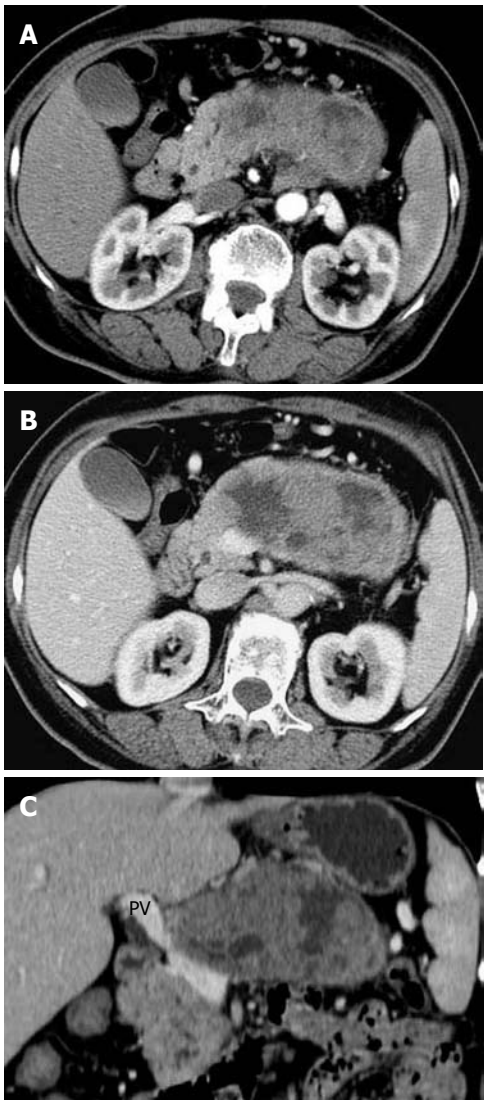

Figure 2 Malignant SPT in a 35-year-old woman with abdominal pain and mass for 6 mo. A: A mass measuring 13 x 5.5 cm was detected on axial MDCT imaging in the pancreatic body. The mass was markedly enhanced and superimposed by abnormal enhancement of tiny vessels at the arterial phase. The interface between the tumor and the adjacent pancreatic parenchyma was blurred in that the tumor apparently infiltrated the surrounding pancreas; B: At the portal venous phase, the axial image demonstrated the heterogeneity of cystic and solid components inside the tumor. The peritumoral capsule was not smooth, which was consistent with capsular invasion. The portal vein was deformed by tumoral invasion; C: Coronal MPVR image showed the tumoral invasion resulting in the narrowing and irregularity of portal vein (PV).

Table 3 Correlation between tumor size and aggressive behaviors in SPTs

Tumor size	Vascular involvement		Metastasis		Capsular invasion	
	Positive	Negative	Positive	Negative	Positive	Negative
< 6 cm	1	10	0	11	0	6
⩾ 6 cm	8	5	[1]3	10	9	4

Fisher's exact test: $P = 0.013$ for vascular involvement; $P = 0.001$ for capsular invasion. [1]2 cases with lymph node metastasis and 1 case with lymph node metastasis and hepatic metastasis.

papillary neoplasm with borderline malignant potential, and solid-papillary carcinoma[14]. To date, more than 700 cases have been reported in the English literature[15]. About 15% are known to present with metastasis or recurrence[3]. However, based on the conventional histopathology, it has been difficult to establish the criteria that are suggestive of aggressive behavior including recurrence and metastasis[14,16-17].

CT is the imaging modality of choice for detection and characterization of SPTs of the pancreas, while the MRI can be more accurate in differentiating the cystic or solid component inside the tumor[7]. If MRI reveals an encapsulated mass lesion with solid and cystic component as well as hemorrhage without obvious internal septations, SPT of the pancreas should be suspected[7]. According to Yu[7], the MRI

Table 4 Vascular invasion on MDCT in comparison with surgical and pathological results in six malignant and two benign SPT cases

MDCT	Surgical and pathological results							
	PV (n = 5[1])	SV (n = 5)	SMV (n = 5)	CA (n = 2)	CHA (n = 3)	PHA (n = 3)	SA (n = 3)	SMA (n = 3)
Axial images	3	1	2	0	3	3	1	0
CTA MIP images	5	3	5	1	3	3	4[2]	3
MPVR images	5	3	4	1	3	3	3	3
A+C+M	5	5	5	2	3	3	3	3

[1]indicating three malignant and two benign SPTs; [2]one of four cases was overestimated; CTA: Computed tomography angiography; MIP: Maxiumum intensity projection; MPVR: Multiplanar volume reformation; A+C+M: Combination of axial images and CTA MIP and MPVR images. PV: Portal vein; SV: Splenic vein; SMV: Superior mesenteric vein; CA: Celiac artery; CHA: Common hepatic artery; PHA: Proper hepatic artery; SA: Splenic artery; SMA: Superior mesenteric artery.

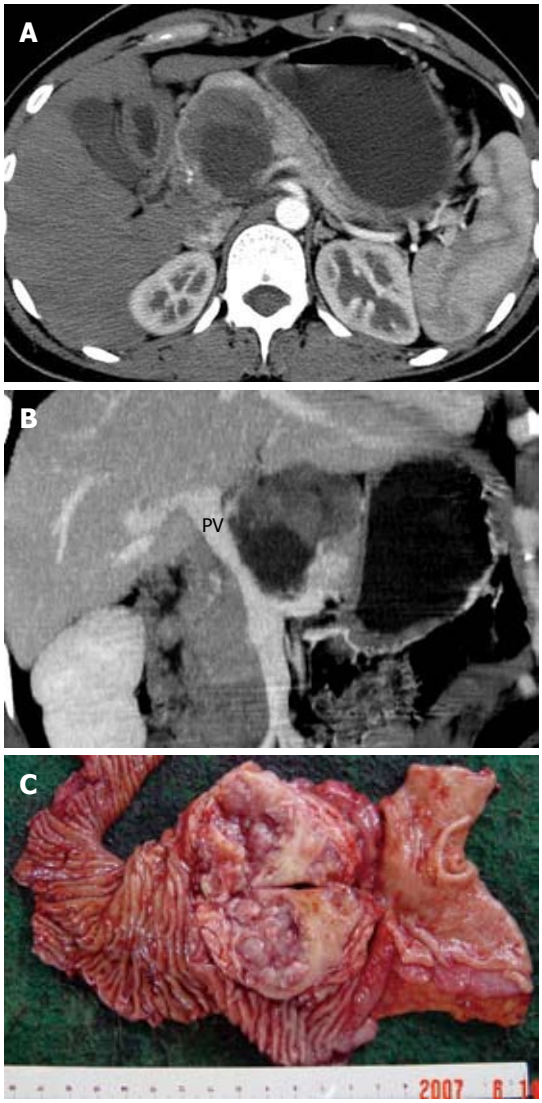

Figure 3 Pathologically confirmed benign SPT in a 30-year-old woman with abdominal pain for about 3 mo. A: A heterogeneously low-attenuation mass was identified in the pancreatic head on the axial MDCT image at the arterial phase. The capsule was irregular and not attached to the adjacent, pancreas implying invasion outside the mass. The peripheral portion of the mass close to the duodenum was enhanced significantly and the duodenum was infiltrated; B: MPVR image revealed the irregularity and narrowing of the portal vein (PV) indicating vascular invasion; C: Invasion into the duodenum was revealed in the gross specimen of the tumor.

findings of SPTs were well correlated with the pathological patterns. Fine needle aspiration biopsy guided by endoscopic ultrasound can help distinguish SPTs from other pancreatic lesions, since the masses are commonly localized in the pancreatic head, thus playing an important role in preoperative planning[18]. Therefore, the imaging modalities are very useful in preoperative assessment of this disease and could provide strong evidence for treatment protocol planning. It is well known that MDCT can provide more accurate delineation of normal and abnormal pancreatic morphology[6]. Furthermore, MDCT can be performed intrinsically for CTA scanning, and the CTA images can be conveniently obtained by post-processing techniques at the dedicated workstation, meanwhile, the coronal and sagittal images can be reformed using axial source images. Thus, axial CT and CTA images can be simultaneously obtained during a single scanning.

The SPT is characterized with imaging features of a well-circumscribed mass, which is surrounded by a clear-depicted peritumoral capsule, and vulnerable to hemorrhage and cystic changes, resulting in the heterogeneously cystic central component and solid periphery. The overview of the clinicopathological characteristics of the SPTs in our series (Table 1) is almost identical to the data reported in the literature[16]. Our MDCT images demonstrated that peripheral and persistent enhancement in solid components occurred in 95.8% (23/24) of all cases. As is well documented in the literature[19], the enhancement of SPTs was not significant in the adjacent pancreatic parenchyma. However, 50% (3/6) of cases in this group with marked enhancement turned out to be malignant. It seems that the hypervascular feature on MDCT could imply an aggressive nature, since half of the malignant cases were categorized as having marked enhancement. Further study is needed to confirm this.

The accuracy of MDCT diagnosis of SPTs was well correlated with the surgical and pathological results (Kappa = 0.6, $P < 0.05$) (Table 2). As a matter of fact, some of the pathologically benign cases with local invasion detected by MDCT in this group might turn out to be malignant during follow-up, as was reported in two cases herein. Although the axial and coronal reformed images, as well as CTA images, could reveal the aggressive behavior of SPTs, the combination of all these images seems to add value to the assessment of the status of tumoral invasion into the adjacent major

vasculature (Table 4). However, statistical analysis was not carried out for the capability of identification of vascular invasion by different images, since the limited number of cases included would bias the results.

In this group, when the mass was more than 6 cm (including 6 cm) in diameter, the tumor was more vulnerable to having aggressive behavior such as metastasis and recurrence. The mean mass size in our series was 5.8 cm. When we set up the threshold of tumor size at 6 cm, the nearby vascular involvement (8 *vs* 1) and peritumoral capsular invasion (9 *vs* 0) were more common in SPTs bigger than 6 cm (including 6 cm), which indicated the biologically aggressive nature revealed by MDCT ($P < 0.05$, Table 3). Interestingly, if the mass was smaller than 6 cm, there was no case categorized as having capsular invasion in this group, which may be wrong if the same size threshold were applied to other groups, since different imaging modalities were employed. As a result of invasion beyond the capsule, invasion into the adjacent pancreatic parenchyma was identified in nine cases, including six malignant cases. Two of the three cases in this settings regarded as benign pathologically had aggressive behavior of vascular involvement and capsular invasion on MDCT, and subsequently presented with local recurrence and hepatic metastasis during follow-up after surgery of the primary tumor. In this context, the findings on MDCT could predict the possible aggressive nature of SPTs of the pancreas, based on our data. Complete surgical resection can be proposed for SPTs and the prognosis is supposed to be excellent[15]. Up to the time of writing, no patient died during the follow-up in our group.

In conclusion, based on our SPT series, local invasion, including vascular and capsular invasion with superimposed spread into the adjacent pancreatic parenchyma and peripancreatic structures and organs, can be accurately depicted by MDCT preoperatively. The findings suggestive of malignancy on MDCT were well correlated with aggressive behavior of the tumor, even in the pathologically benign cases. Therefore, the value of imaging features implying aggressive behavior of SPTs needs to be emphasized; it seems that the imaging findings are predictive of the malignant potential associated with aggressive nature, and are probably beneficial to the patient's surgical protocol planning.

COMMENTS

Background

The solid-pseudopapillary tumor (SPT) of the pancreas is rare, mainly occurring in young women. To date, more than 700 cases have been reported in the English literature. About 15% are known to present with metastasis or recurrence. However, based on conventional histopathology, it has been difficult to establish the criteria that are suggestive of the aggressive behavior including recurrence and metastasis.

Research frontiers

No pathological factors including mitotic rate, nuclear pleomorphism and vascular invasion were found to correlate with the prognosis of SPT. Also, the histopathological features suggestive of malignant potential were non-specific. Aggressive behavior may not be completely excluded, even in the absence of pathological features suggesting malignant potential. CT is the imaging modality of choice for detection and characterization of SPTs of the pancreas while MRI can be more accurate in differentiating the cystic from solid component inside the tumor.

Innovations and breakthroughs

To the best of our knowledge, there is no published literature regarding the imaging features implying the malignant potential of SPTs of the pancreas on multi-detector row computed tomography (MDCT). In our series, when the size of the tumor was greater than 6 cm (including 6 cm), the possibilities of vascular (8 *vs* 1) and capsular invasion (9 *vs* 0) increased significantly ($P < 0.05$). Two pathologically benign cases with vascular invasion and disrupted capsule on MDCT presented with local recurrence and hepatic metastases during follow-up, about 1 year after resection of the primary tumors. Vascular and capsular invasion with superimposed spread into the adjacent pancreatic parenchyma and nearby structures in SPTs of the pancreas could be accurately revealed by MDCT preoperatively. These imaging findings could be predictive of the malignant potential associated with aggressive behavior of the tumor, even in the pathologically benign cases.

Applications

The findings suggestive of the malignancy on MDCT are well correlated with aggressive behavior of the tumor, even in the pathologically benign cases. Therefore, the value of imaging features implying aggressive behavior of SPTs needs to be emphasized. It seems that the imaging findings are predictive of the malignant potential associated with aggressive nature and are beneficial to the patient's surgical protocol planning.

Terminology

"Solid pseudopapillary": Encompasses the two most conspicuous histological features, which are also depicted macroscopically, the cystic center and the solid periphery of the mass. "MDCT": Multiple detectors applied to CT. This modality can improve the scanning speed and spatial resolution dramatically. Furthermore, MDCT can be performed intrinsically for CT angiography scanning. "Aggressive behavior": The tumor with aggressive behavior means that it has local invasion, local recurrence, or metastasis.

Peer review

This is a retrospective study of the radiological features of SPT upon MDCT in 24 consecutive cases. Radiological changes have been correlated with operative and pathological findings. These imaging findings could be predictive of the malignant potential associated with aggressive behavior of the tumor, even in pathologically benign cases.

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ORIGINAL ARTICLES

Detachment of esophageal carcinoma cells from extracellular matrix causes relocalization of death receptor 5 and apoptosis

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Abstract

AIM: To investigate the effect of detachment of esophageal cancer cells from extracellular matrix on the localization of death receptor 5 (DR5) and apoptosis.

METHODS: Anchorage-dependent EC9706 cells of esophageal squamous cell carcinoma were pretreated or not treated with brefeldin A. Detached cells were harvested by ethylenediaminetetraacetic acid digestion. Expression and localization of DR5 in these cells were determined by immunocytochemical and immunofluorescence assays, as well as flow cytometry analysis. Apoptosis of EC9706 cells was detected by flow cytometry after stained with fluorescein isothiocyanate-labeled annexin V/propidium iodide. Activation of caspase 8 was detected by Western blot analysis.

RESULTS: Immunocytochemical assay indicated that DR5 was predominantly perinuclear in adherent cells but was mainly localized in cell membrane in detached cells. In addition, immunofluorescence assay also confirmed the above-mentioned results, and further demonstrated that DR5 was present in the form of coarse granules in detached cells, but in the form of fine granules in adherent cells. Cytometry analysis revealed higher levels of DR5 expression on the surfaces of brefeldin-A-untreated cells than on the surfaces of brefeldin-A-treated cells, but brefeldin A treatment did not affect the total DR5 expression levels. Moreover, nocodazole did not influence the extracelluar DR5 expression levels in EC9706 cells. Apoptosis assay revealed that detached cells were more sensitive to DR5 antibody-induced apoptosis than adherent cells. Western blotting showed that caspase 8 was activated in temporarily detached cells 4 h earlier than in adherent cells.

CONCLUSION: Progress from adhesion to detachment of EC9706 cells causes DR5 relocalization, and promotes cytoplasmic translocation of DR5 to cell surfaces *via* a Golgi-dependent pathway. Moreover, it might also result in DR5 aggregation to render apoptosis of detached cells.

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Key words: Translocation of death receptor 5; Cell detachment; Esophageal carcinoma; Anoikis; Apoptosis

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INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand

(TRAIL) was identified in 1995 based on its sequence homology to FasL/Apo1L and TNF[1]. TRAIL is a type Ⅱ membrane protein and can be cleaved from the cell surface to form a soluble ligand. Both membrane-bound and soluble TRAIL can rapidly induce apoptosis in a variety of tumor cells and transformed cells, with minimal adverse effects on normal cells[1,2]. In addition, it was reported that recombinant human TRAIL (rhTRAIL), in combination with chemotherapy or radiotherapy, has synergistic effects on several types of human cancer and can overcome resistance to both chemotherapy and radiotherapy[2]. Due to its highly selective tumoricidal activity, TRAIL is a promising agent for cancer therapy and mediates apoptotic effects by binding to its agonistic death receptors as a homotrimer. Several TRAIL receptors have been discovered to date, including TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4. TRAIL-R1 and TRAIL-R2, which are death receptors, commonly known as DR4 and DR5, respectively, transduce TRAIL-mediated death signals to the intracellular apoptotic machinery. However, TRAIL-R3 and TRAIL-R4 are designated as anti-apoptotic decoy receptors that antagonize TRAIL-induced apoptosis[2]. It was reported that DR5 is probably the main TRAIL death receptor[2,3] because it exhibits a considerably higher affinity for TRAIL than DR4 in physiological conditions (37℃)[4], and may play a more prominent role than DR4 in mediating apoptotic signals emanating from TRAIL in cells expressing both death receptors[5]. Thus, DR5 is a good potential target for anti-tumor therapy.

Since TRAIL and agonistic antibodies against DR5 activate membrane-bound DR5 to trigger apoptosis, cellular localization sites of DR5 directly affect TRAIL-induced apoptosis[2,3]. Therefore, it is very important to elucidate the cellular localization of DR5 and the mechanisms underlying the regulation of DR5 expression and transport. Cellular localization of DR5 has been partially determined. There are numerous DR5s in cytoplasm and a few on the cell membrane of human melanoma cells, but DR5 is not present in nuclei[6]. Recently, Laguinge *et al*[7] reported that DR5 expression is increased in detached human colorectal carcinoma cells and DR5 mediates anoikis through a caspase 8-dependent pathway. In the development and progress of malignant tumors, as detachment from primary tumor is an obligatory step in metastasis, tumor cells must detach from their substrates at a distant site to create metastasis. Furthermore, high levels of TRAIL have been reported in tumor-infiltrating lymphocytes of cancer patients, thus high levels of DR5 in tumor cells will decrease the survival and metastasis of detached cells. Therefore, low levels of DR5 are frequently expressed in metastasized cells[8,9]. To date, the mechanisms underlying the expression and translocation of DR5 are still unclear.

In the present study, we investigated the expression, relocalization, and translocation of DR5 in esophageal cancer cells during the process of cell detachment. Our data suggest that detachment of EC9706 cells from extracellular matrix cause DR5 relocalization, promotes cytoplasmic translocation of DR5 to cell surfaces *via* a Golgi-dependent pathway, and might also result in DR5 aggregation. Our findings provide new insights into the mechanisms underlying the regulation of intracellular localization and translocation of DR5 and also indicate the potential clinical applications of DR5 antibodies and TRAIL in anticancer therapy.

MATERIALS AND METHODS

Materials

Neutralizing monoclonal antibody 366 EC and functional monoclonal antibody mDRA-6 with apoptotic activity against DR5 were prepared as previously described[5,10]. Rabbit anti-mouse SP kit and DAB kit were from Zymed Laboratories (San Francisco, CA, USA). Endogenous peroxidase blocking kit was obtained from Vector Laboratories (Burlingame, CA, USA). Goat anti-human caspase-8 antibody was from R&D systems (Minneapolis, MN, USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG1, horseradish peroxidase (HRP)-conjugated goat anti-mouse and rabbit anti-goat IgG1, and ECL plus Western blotting detection system were from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Annexin V-FITC/PI apoptosis detection kit was from BD Pharmingen (San Diego, CA, USA). Polyvinylidene difluoride membrane was from Millipore Corp (Bedford, MA, USA). Fetal bovine serum was from Tianjin Haoyang Biological Manufacture Co., Ltd (Tianjin, China). RPMI-1640 medium, trypsin, and ethylenediaminetetraacetic acid (EDTA) were from Gibco BRL (Gaithersburg, MD, USA). Brefeldin A and nocodazole were from Sigma (St. Louis, MO, USA). Other chemicals used were of analytical reagent grade.

Cell culture

Esophageal cancer cell line EC9706 was obtained from Cancer Institute, Chinese Academy of Medical Sciences (Beijing, China). EC9706 cells were cultured in RPMI-1640 medium supplemented with 100 mL/L fetal bovine serum, 50 IU/mL penicillin, and 50 mg/L gentamycin at 37℃ under an atmosphere containing 50 mL/L CO_2. The medium was changed every 2 d until 80%-90% confluence was achieved. The cells were harvested by 0.3 g/L trypsin-EDTA digestion for subculture or to prepare a single-cell suspension for subsequent experiments.

Detection of DR5 expression by flow cytometry

To detect the cell-surface expression of DR5, EC9706 cells (10^5/well) were seeded in a 24-well plate and incubated for 10 h at 37℃ under an atmosphere containing 50 mL/L CO_2. Adherent cells were incubated in a medium containing brefeldin A at a final concentration of 10 mg/L, or only in the culture medium, for 30 min, and then trypsinized to obtain a single-cell suspension for subsequent experiments. The adherent cells were incubated in the medium containing nocodazole at a final concentration of 10 mg/L, or only

in the culture medium, for 8 h, followed by trypsinization to obtain a single-cell suspension. The cells were washed with a staining buffer (Hank's solution into which 1 g/L BSA and 1 g/L NaN_3 were added). One portion of the cells was used for extracellular staining. These cells were stained on ice with 4 mg/L 366EC antibody in 100 μL of staining buffer for 40 min, washed twice with PBS, and then incubated on ice with FITC-IgG for 40 min, avoiding direct light exposure. After two final washings with PBS, the cells were fixed in a 10 g/L paraformaldehyde (PFA) solution and finally resuspended in 500 μL PBS. Flow cytometry was performed using a FACSCalibur cytometer (BectonDickinson, San Jose, CA, USA). A minimum of 1×10^4 cells per sample was interrogated and the data were analyzed using the CellQuest software (BectonDickinson). The negative control sample used for flow cytometry experiments was treated with normal mouse serum instead of 366EC antibody. All experiments were repeated at least three times.

In order to measure DR5 intracellular protein levels as well as extracellular expression, the remaining cells were permeabilized by methanol and 1 g/L saponin after fixation using 10 g/L PFA. The cells were stained following the same procedure as the extracellular staining except for the lack of a final fixation step in 10 g/L PFA. Total DR5 expression level was analyzed by flow cytometry as described above.

Intracellular localization of DR5

For immunocytochemical staining, the EC9706 cells were cultured as a monolayer in six-well dishes containing sterilized cover-slips at 37℃ for 12 h. The adherent cells were treated with brefeldin A at a final concentration of 10 mg/L, or with culture medium only, for 30 min. The adherent cells plated in six-well dishes were digested by EDTA buffer to obtain a single-cell suspension. After two final washings with PBS, the detached cells were spun on coverslips. After treatment, all cells on the coverslips were fixed in cold methnol: water (1:1) for 10 min and dried for 5 min at room temperature. Endogenous peroxidase was blocked with 3 mL/L H_2O_2 in a PBS solution for 30 min. The coverslips were then incubated with avidin and biotin blocking solutions, pre-incubated with 50 mL/L normal mouse serum and 10 g/L bovine serum albumin (BSA) in PBS for 15 min, and then incubated with primary antibodies (4 mg/L 366EC) at 37℃ for 1 h. After washing with PBS, the coverslips were incubated with biotinylated goat-antimouse antibody at room temperature for 40 min, and then with streptavidin-peroxidase at room temperature for 15 min. Peroxidase activity was observed by incubating the slides for 3 min in a DAB solution. After washed several times, the coverslips were counterstained with hematoxylin (or not counterstained), dehydrated with ethanol, rinsed in xylene, and then mounted with gum for microscopic examination and photography. The negative control sample was treated with normal mouse serum instead of 366EC antibody.

For immunofluorescence staining, the cells were treated as described above. After the cells were incubated with primary antibody, secondary antibody FITC-IgG diluted at 1:200 was added, and incubated at 4℃ in the dark and at room temperature for 1 h, respectively. The staining specificity was analyzed under an Axioskop 2 plus fluorescence microscope (Carl Zeiss, Oberkochen, Germany) using a 40 × objective lens. Image capture was performed with a Spot RT color CCD camera and the Spot RT software (Diagnostic Instruments, Sterling Heights, MI, USA). All experiments were repeated at least three times. The negative control sample was treated with non-immune normal rabbit IgGs instead of primary antibodies.

Analysis of apoptosis

Apoptosis was evaluated using an Annexin V-FITC apoptosis detection kit. Briefly, EC9706 cells (10^5/well) were seeded in a 24-well tissue culture plate and incubated for 10 h. The cells were randomly divided into adhesion group, detachment group, and negative control group. Cells in the adhesion group were treated with 2 μg/mL mDRA-6 antibody for 12 h, cells in the detachment group were trypsinized to prepare a single-cell suspension (detached cells) and then detached cells were treated with 2 mg/L mDRA-6 antibody for 12 h, and cells in the negative control group were incubated in a medium without mDRA-6 antibody for 12 h. Subsequently, floating and adherent cells in the medium were collected and centrifuged at 1800 r/min for 6 min at 4℃. Cell pellets were washed twice with cold PBS and then resuspended in a binding buffer at a concentration of 1×10^6 cells/mL, and 100-μL aliquots of this cell suspension (1×10^5 cells) were then transferred to a 5-mL culture tube. Using an Annexin V-FITC apoptosis detection kit, the cells were stained with AnnexinV-FITC and propidium iodide (PI) according to the manufacturer's instructions. Five microliters of annexin V-FITC and 5 μL of PI were added to each 100-μL aliquot of the above-mentioned cell suspension, and the cells were gently vortexed and incubated for 15 min at room temperature in the dark. Each sample, to which 400 μL of 1 × binding buffer was added, was analyzed using a FACSCalibur cytometer within 1 h. A minimum of 1×10^4 cells was detected in each flow cytometry sample and the data were analyzed using the CellQuest software.

Western blot analysis

To assess the caspase-8 activation, after required treatments, cells (3×10^6) were washed once with PBS and lysed in the sample buffer (200 μL) for SDS-PAGE and immediately boiled for 4 min. Each sample was subjected to 15 % SDS-PAGE, and proteins separated on the gel were subsequently electrotransferred onto a polyvinylidene difluoride membrane which was blocked with 50 g/L non-fat dry milk in TBS-T (20 mmol/L Tris-HCl at pH 7.4, 8 g/L NaCl, and 1 mL/L Tween 20) for 2 h at room temperature. The membrane was then incubated with goat anti-human caspase-8 antibody in TBS-T containing 50 g/L non-fat dry milk at 4℃ overnight, washed three times with TBS-T and probed

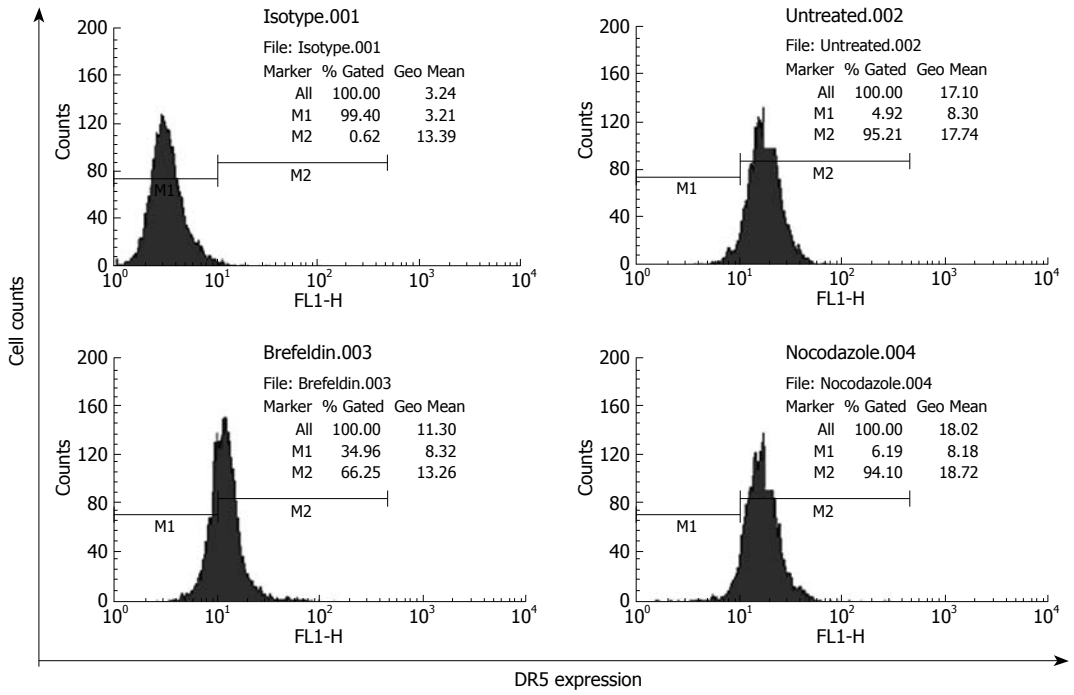

Figure 1 Analysis of DR5 extracellular expression in EC9706 cells by flow cytometry. Isotype.001: Isotype control; Untreated.002: Extracellular expression of DR5 in the cells pretreated without brefeldin A; Brefeldin.003: Extracellular expression of DR5 in the cells pretreated with brefeldin A; Nocodazole.004: Extracellular expression of DR5 in the cells pretreated with nocodazole for 8 h and without brefeldin A.

with peroxidase-conjugated rabbit anti-goat antibody at room temperature for 2 h. After washing four times with TBS-T, the protein was observed using the ECL Plus Western blotting detection system according to its manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SAS® 6.12 (SAS Institute, Cary, NC, USA). All values were expressed as mean ± SD. The F test was used to compare pairs of means. $P < 0.05$ was considered statistically significant.

RESULTS

DR5 expression in EC9706 cells

In the progress of malignant tumors, detachment is very important to the metastasis of tumor cells and tumor cells must detach from their substrates frequently at a distant site to create metastasis. As the expression level of DR5 receptors on cell surfaces directly affects TRAIL-induced apoptosis, it is thus essential to investigate the relation between DR5 expression on cell surfaces and cell detachment. To determine DR5 expression on the surfaces of detached cells, detached cells were harvested after pretreatment with or without brefeldin A, which can specifically disrupt the functions of Golgi apparatus[11] and block proteins trafficking to cell membranes. The detached cells labeled with FITC-IgG were detected by flow cytometry. The DR5 expression rate on cell surfaces was 96.21% in brefeldin A-untreated EC9706 cells. However, it was decreased to 66.25% ($P < 0.01$) and the expression intensity was decreased from 17.34 to 13.26 ($P < 0.05$) in brefeldin-A-treated cells (Figure 1), suggesting that transformation of EC9706 cells from a spreading adherent to a detached status is accompanied with translocation of DR5 to the cell surface.

Brefeldin A treatment did not change the total DR5 expression, indicating that detachment does not influence DR5 synthesis, and increased DR5 expression on the cell surface could not be simply explained by DR5 protein synthesis. Since cytoplasmic DR5 is mainly localized within the Golgi apparatus[6] and brefeldin A can specifically inhibit the functions of the Golgi network[11], the present results indicate that Golgi network is involved in DR5 translocation from the cell interior to the plasma membrane, and DR5 shuttles to the cell surface in a classic protein secretory route. It has been reported that DR5 translocation from the Golgi to plasma membrane occurs along microtubules, and nocodazole inhibits Fas translocation to plasma membrane by disrupting microtubules[12,13]. In the present study, nocodazole should have attenuated DR5 expression on the surface of detached cells, but DR5 expression remained unchanged in nocodazole-treated cells (Figure 1), indicating that microtubules are not involved in DR5 translocation.

The total DR5 expression level was 10-fold higher than the cell surface expression of DR5 in brefeldin-A-untreated EC9706 cells (Figure 2), demonstrating that the majority of total cellular DR5 was sequestered within intracellular compartments and the minority was associated with the plasma membrane.

DR5 localization in EC9706 cells

To further investigate the cellular localization and confirm the apparent translocation of DR5 from cell interior to plasma membrane, EC9706 cells were examined with immunostaining. In adherent cells, the results of both immunocytochemical and immunofluorescence staining showed that DR5 had a predominantly perinuclear

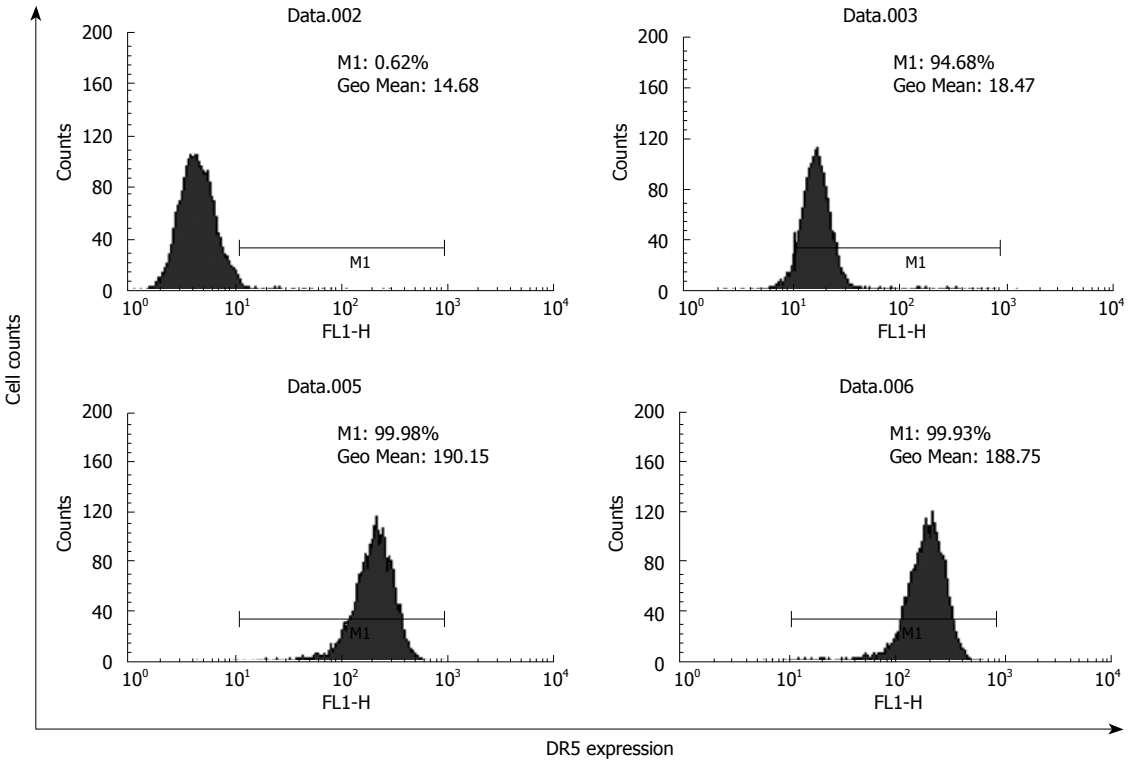

Figure 2 Analysis of total DR5 and extracellular expression in EC9706 cells by flow cytometry. Data.002: Isotype control; Data.003: Extracellular expression of DR5 in the cells pretreated without brefeldin A; Data.005: Total DR5 expression in the cells pretreated without brefeldin A; Data.006: Total DR5 expression in the cells pretreated with brefeldin A for 30 min before trypsinization.

localization but no nuclear localization (Figures 3A and 4A) and DR5 localization was not significantly different between brefeldin-A-treated and untreated cells (Figure 3A and B and Figure 4A and B). In detached cells, DR5 was predominantly localized in cytoplasm but not in nuclei (Figure 3C and D and Figure 4C and D). Furthermore, in brefeldin-A-untreated detached cells, DR5 tended to localize in the cell membrane and became exactly consistent with aggregation or capping of DR5 (Figure 4C). However, in brefeldin-A-treated cells, DR5 exhibited perinuclear localization (Figures 3D and 4D) and diffused (Figure 4D). These findings suggest that loss of cell adhesion enhanced DR5 translocation to the membrane of detached cells, leading to DR5 relocalization and aggregation.

Apoptosis in EC9706 cells

Since binding of DR5 agonist antibody mDRA-6 to DR5 triggers apoptotic cascades[10], we reasoned that by increasing cell surface expression of DR5, detached cells might become more susceptible to apoptosis induced by mDRA-6. Indeed, apoptosis induced by mDRA-6 was significantly increased in detached cells (Figure 5). The ability of detachment to sensitize EC9706 cells to apoptosis induced by mDRA-6 was inhibited by brefeldin A, suggesting that DR5 trafficking from cell interior to surface is necessary for detachment sensitization of EC9706 cells to mDRA-6-induced apoptosis. Interestingly, the spontaneous apoptotic rate for detached cells was higher than that for adherent cells ($P < 0.05$), demonstrating that increased cell surface expression of DR5 in detached cells is functional in transducing death signals and loss of cell adhesion may trigger apoptotic cascades *via* DR5 aggregation.

Activation of capsase-8 in EC9706 cells

It has been shown that TRAIL and anti-DR4/DR5 antibodies could trigger apoptotic signaling pathway of death receptor by activating caspase 8[2,10], which is implicated in apoptosis induced by cell detachment[7,14]. To characterize the signaling pathways through which detachment treatment substantially enhances mDRA-6-induced killing of EC9706 cells, activation of caspase 8 was determined by Western bloting in EC9706 cells. Consequently, time-dependent activation of caspase 8, as determined by decreased procaspase 8 (p55/53) levels and cleavage of caspase 8 (p43/p41), was observed after mDRA-6 treatment in spreading and temporarily detached EC9706 cells, respectively. Caspase 8 was activated 4 h earlier in temporarily detached cells than in spreading cells (Figure 6). However, even if the level of spontaneous apoptosis was much higher in temporarily detached EC9706 cells than in spreading EC9706 cells, caspase 8 activation was not detectable by Western bloting (data not shown), suggesting that activation of caspase 8 induced by cell detachment *via* DR5 oligomerization activation is very weak and is thus difficult to be assessed by Western bloting.

DISCUSSION

Since DR5 is the key death receptor of TRAIL[2-4], it is

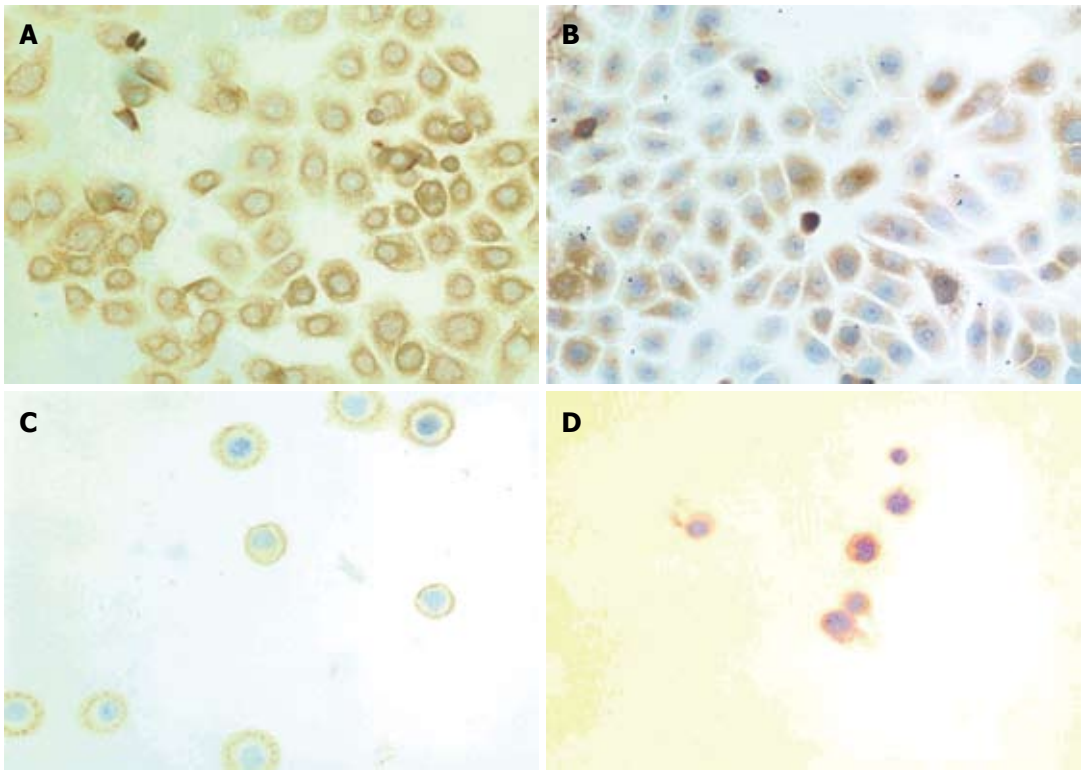

Figure 3 Immunostaining showing expression and localization of DR5 in EC9706 cells. A: Adherent cells pretreated without brefeldin A; B: Adherent cells pretreated with brefeldin A for 30 min; C: Detached cells not pretreated with brefeldin A before trypsinization; D: Detached cells pretreated with brefeldin A for 30 min before trypsinization (x 400).

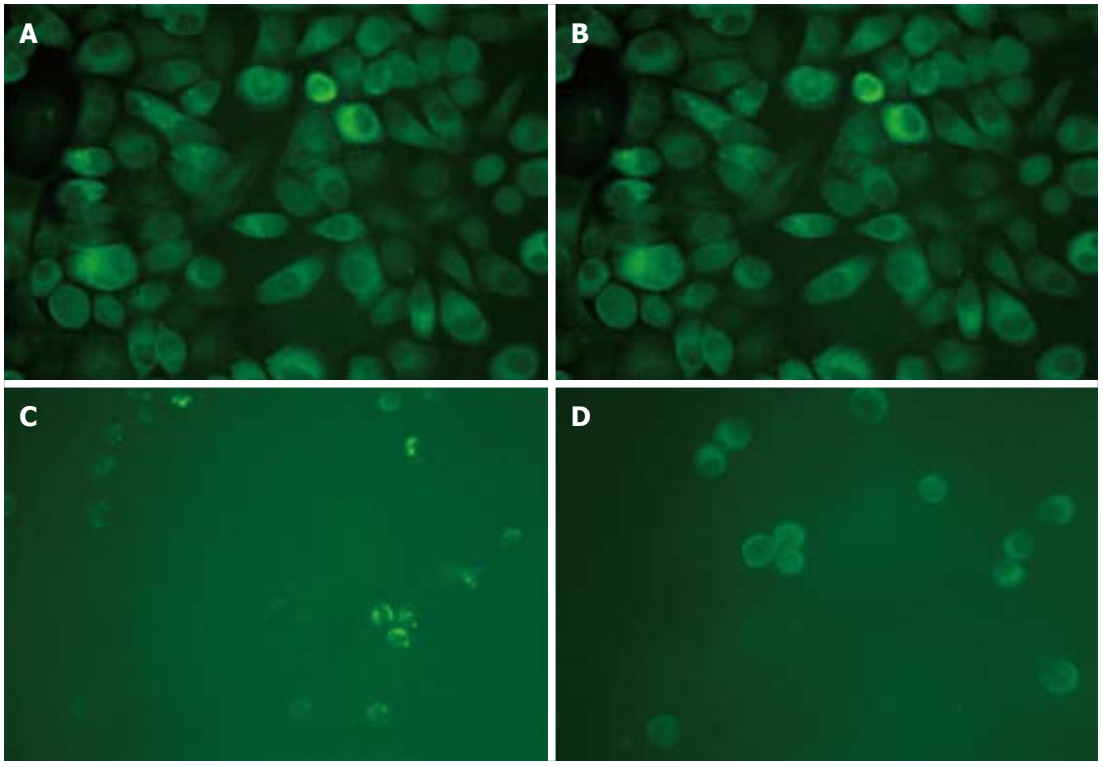

Figure 4 Immunofluorescence showing expression and distribution of DR5 in EC9706 cells. A: Adherent cells treated without brefeldin A; B: Adherent cells treated with brefeldin A before trypsinization; C: Detached cells not treated with brefeldin A; D: Detached cells treated with brefeldin A for 30 min before trypsinization (x 400).

very important to investigate its cellular expression and localization at the protein level. DR5, mainly localized in Golgi apparatus of cytoplasm, is a type of membrane protein[6] and translocated to cell membrane through the Golgi network. Brefeldin A can specifically inhibit the function of the Golgi apparatus[11]. This study showed

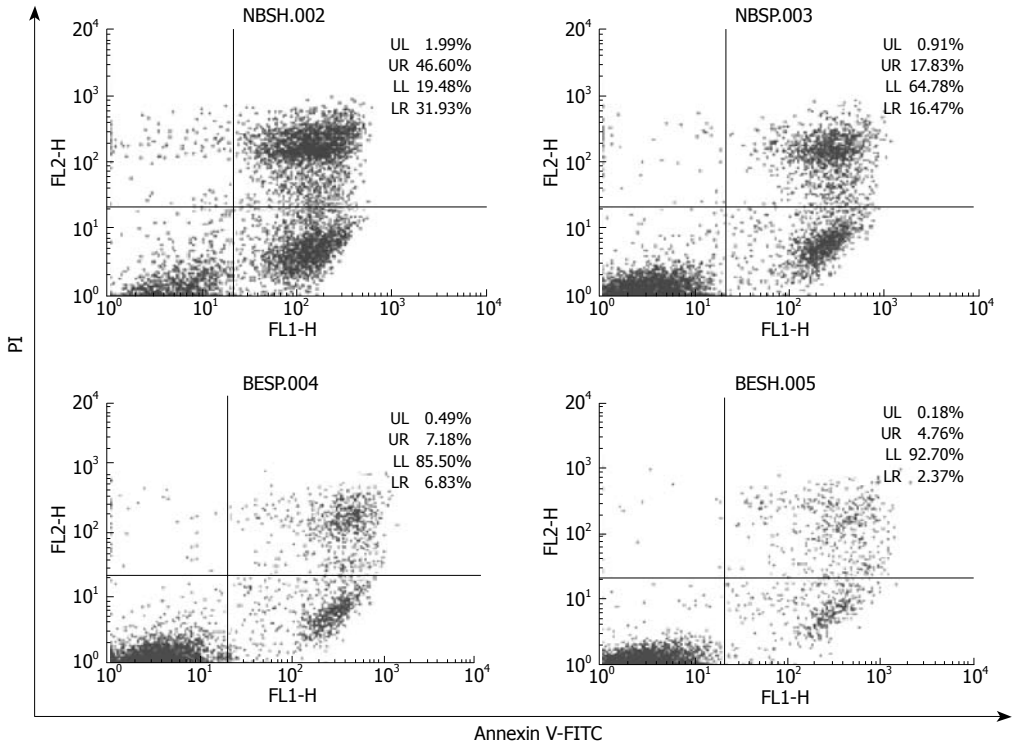

Figure 5 Flow cytomery showing apoptosis of EC9706 cells induced by anti-DR5 agonistic antibody. NBSH: Detached cells incubated with 2 mg/L mDRA-6 antibody for 12 h; NBSP: Spreading cells incubated with 2 mg/L mDRA-6 antibody for 12 h; BESH: Detached cells incubated in the medium without mDRA-6 antibody for 12 h; BESP: Spreading cells incubated in the medium without mDRA-6 antibody for 12 h.

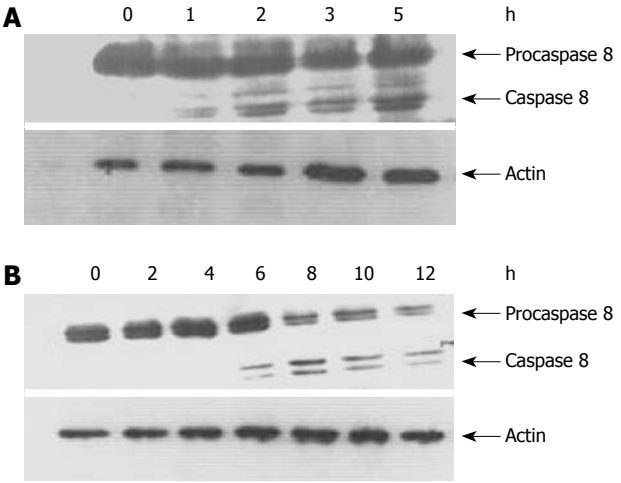

Figure 6 Activation of caspase 8 in EC9706 cells. A: After trypsinization, temporally detached EC9706 cells were incubated with agonist antibody mDRA-6 for an indicated time at 37℃; B: Spreading EC9706 cells were incubated with agonist antibody mDRA-6 for an indicated time at 37℃. Cell lysates were prepared and Western blotting was performed to analyze caspase 8 activation. The position of procaspase 8 and the active subunits were indicated. Western bloting for actin was utilized as the control of an equal sample loading.

that after EC9706 cells were treated with brefeldin A, the proportion of cells expressing DR5 on cell surface was significantly reduced in detached EC9706 cells, indicating that cell detachment is accompanied with translocation of DR5 from the cytoplasm to cell membrane (Figure 1), but a slight translocation occurred since DR5 was mainly localized in the cytoplasm (Figure 2), and total protein expression of DR5 did not increase in detached cells. However, it was recently reported that suspension culture enhances gene transcription and protein synthesis in DR5, but cell detachment does enhance DR5 expression on the cell surface[7]. Cell detachment from extracellular matrix had no effect on DR5 protein synthesis and the reason for this discrepancy was that cell detachment was temporary. To probe into DR5 translocation, morphological changes were observed with immunostaining in the present study. DR5 exhibited predominantly perinuclear localization in spreading EC9706 cells, while DR5 receptors were localized near the cell membrane of detached cells (Figures 3 and 4), revealing that cell detachment is mainly responsible for intracellular relocalization of DR5 and promotes translocation of DR5 to the cell membrane. It has been shown that DR5 receptors are mainly localized in the plasma membrane of some suspension cells[15], and in the cytoplasm of some spreading cells[6,16], suggesting that cellular localization of DR5 is closely related to the growth pattern and adhesion status of cells.

It was reported that the Golgi complex and cytoskeletal system are directly involved in translocation of membrane proteins and secretory proteins[11,13,17]. Cell detachment influences cellular localization of DR5 and promotes translocation of DR5 to the cell membrane, indicating that the cytoskeleton may be involved in cellular localization and translocation of DR5. It has been shown that glycyl-chenodeoxycholic acid may enhance Fas expression on the surface of human liver cells, but nocodazole which can disrupt microtubules, inhibits the function of bile acids, suggesting that microtubules are

involved in translocation of Fas[13]. However, results of the current study show that there was no difference in DR5 expression rate and intensity between nocodazole-treated and untreated esophageal cancer cells (Figure 1), demonstrating that microtubules are not implicated in DR5 translocation. The reason for this discrepancy still remains unclear and the inconsistent results might be attributed to the types of death receptors, cells, and stimulating period of time.

It has been shown that DR5 expression on the surface of tumor cells is closely related to the sensitivity of tumor cells to TRAIL-induced apoptosis[2,3]. Therefore, increased DR5 expression on the cell surface might be accompanied with increased cell sensitivity to TRAIL-induced apoptosis. mDRA-6 is an anti-DR5 agonist antibody and exhibits tumoricidal activities by inducing apoptosis[10]. This study demonstrated that when spreading esophageal cancer cells were transformed to detached cells, the rate of mDRA-6-induced apoptosis was increased by 44.23% (Figure 5). Although this was definitely attributed to the high cell-surface-expression levels of DR5 caused by cell detachment, it was more likely due to the aggregation of DR5. Loss of adhesion in esophageal cancer cells was found to be responsible for the granular distribution of DR5 in these cells (Figures 3 and 4), indicating that cell detachment leads to DR5 aggregation. Moreover, it was reported that DR5 aggregation might cause ligand-independent activation of DR5, thus resulting in activation of caspase 8 and consequently apoptosis[18]. It was also reported that detachment could activate caspase-8, leading to anoikis[14]. It has been recently shown that activation of caspase-8 mediated by detachment contributes prominently to DR5[7]. In this study, the spontaneous apoptotic rate for detached EC9706 cells was higher than that for adherent cells ($P < 0.05$) (Figure 5), and caspase 8 was activated 4 h earlier in temporarily detached cells than in spreading detached cells in the presence of DR5 agonist antibody (Figure 6), suggesting that cell detachment may lead to activation of caspase 8 due to DR5 aggregation. However, Western blot analysis revealed that no activated caspase 8 was detected in temporally detached EC9706 cells in the absence of DR5 agonist antibody (Figure 6), and it seems that the period of cell detachment was not long enough, so that activation of caspase 8 induced by DR5 oligomerization was too weak to be detected by Western bloting. DR5 oligomerization activation may exist in detached cells, thus effectively reducing the threshold of apoptosis, which increases the apoptotic sensitivity of detached cells to DR5 agonist antibody.

Cellular localization of DR5 may influence cell survival, and may change from cell adhesion to cell detachment. Since most types of human cells exhibit adhesion growth, DR5 receptors are mainly located in the cytoplasm, and only a few DR5s are present on the cell surface. Cytoplasmic localization of DR5 effectively reduces its interaction with TRAIL, which may lead to apoptosis, thus leading to cell survival. Furthermore, high levels of TRAIL have been reported in tumor-infiltrating lymphocytes of cancer patients[19], and DR5 maybe highly express on the surface of detached tumor cells. Thus, interaction between tumor and immune cells may increase apoptosis of metastatic cells, and inhibit metastasis of tumor cells. Consequently, low expression of DR5 augments tumor metastasis. In addition, if DR5 receptors are mainly located in the cytoplasm of adherent cells, the localization of DR5 may lead to resistance of tumor cells to TRAIL-induced apoptosis and prevent tumor cells from TRAIL-induced apoptosis. However, some drugs can up-regulate DR5 expression and enhance translocation of DR5 to the cell membrane[18,20], and these drugs may increase the sensitivity of tumor cells to TRAIL-induced apoptosis and enhance the efficacy of TRAIL-induced apoptosis. Thus, TRAIL or agonistic anti-DR5 antibody in combination with subtoxic doses of chemotherapeutic agents and radiotherapy may provide a promising effective therapeutic strategy for resistant tumors.

In summary, detachment of EC9706 cells from extracellular matrix causes DR5 relocalization, promotes cytoplasmic translocation of DR5 to the cell surface *via* a Golgi-dependent pathway, and also results in DR5 oligomerization and apoptosis. Our findings provide new insights into the mechanisms underlying the regulation of intracellular localization and translocation of DR5, and also show the potential clinical applications of DR5 antibodies and TRAIL in anticancer therapy. How cellular detachment influences DR5 translocation and aggregation needs to be further studied. Furthermore, additional works are needed to determine whether DR5 expression varies with the adherent phase of cells in all solid tumors.

COMMENTS

Background

DR5, the main agonist death receptor of TRAIL, is a good potential target for anti-tumor therapy. Since TRAIL and agonistic antibody against death receptor 5 (DR5) can activate membrane-bound DR5 to trigger apoptosis, cellular localization of DR5 receptors directly affects TRAIL-induced apoptosis. In the development and progress of malignant tumors, detachment from primary tumor is an obligatory step in metastasis; tumor cells must detach from their substrates at a distant site to create metastasis. To date, DR5 expression caused by cell detachment remains poorly understood. In the present study, the effect of cell detachment on DR5 relocalization and translocation in esophageal carcinoma cells was observed.

Research frontiers

This study was focused on the relocalization and translocation of DR5 and apoptosis during the process of cell detachment.

Applications

This study showed that detachment of EC9706 cells from extracellular matrix caused DR5 relocalization, promoted cytoplasmic translocation of DR5 to the cell surface *via* a Golgi-dependent pathway, and also resulted in DR5 oligomerization. These findings will provide new insights into the mechanisms underlying DR5 translocation regulation and anoikis, and also indicate the potential clinical applications of DR5 antibodies and TRAIL in anticancer therapy.

Peer review

The authors investigated the potential effect of detachment of esophageal squamous carcinoma cells from extracellular matrix on the localization and translocation of DR5, and the results demonstrate that detachment of EC9706 cells from extracellular matrix could cause DR5 relocalization, promote cytoplasmic translocation of DR5 to the cell surface *via* a Golgi-dependent pathway, and result in DR5 oligomerization. The study was well designed with interesting and informative findings.

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BRIEF ARTICLES

Correlation analysis of celiac sprue tissue transglutaminase and deamidated gliadin IgG/IgA

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Abstract

AIM: To indirectly determine if tissue transglutaminase (tTG)-specific T cells play a crucial role in the propagation of celiac disease.

METHODS: Anti-deamidated gliadin peptide (DGP) and anti-tTG IgA and IgG were measured in the sera of celiac patients (both untreated and treated). The correlations were determined by Spearman's rank correlation test.

RESULTS: In celiac patients, we found a very significant correlation between the production of DGP IgA and IgG (r = 0.75), indicating a simultaneous and ongoing production of these two isotypes reminiscent of oral vaccination studies. However, there was far less association between the production of tTG IgA and tTG IgG in celiac patients (r = 0.52). While tTG IgA was significantly correlated with DGP IgA (r = 0.80) and DGP IgG (r = 0.67), there was a weak correlation between production of anti-tTG IgG and the production of anti-DGP IgA (r = 0.38) and anti-DGP IgG (r = 0.43).

CONCLUSION: These data demonstrate that the production of anti-tTG IgA is directly correlated to the production of anti-DGP IgG and IgA, whereas anti-tTG IgG is only weakly correlated. This result therefore supports the hapten-carrier theory that in well-established celiac patients anti-tTG IgA is produced by a set of B cells that are reacting against the complex of tTG-DGP in the absence of a tTG-specific T cell.

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Key words: Celiac disease; Tissue transglutaminase; Deamidated gliadin peptide; Correlation; IgG; IgA

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INTRODUCTION

Celiac disease is a gluten-sensitive disease that afflicts primarily the small bowel, resulting in the shortening of villi, increased numbers of intraepithelial lymphocytes, and crypt hyperplasia[1,2]. One unique feature of celiac disease that is utilized as a diagnostic and screening tool is the production of IgA specific for tissue transglutaminase (tTG) that circulates in the blood[3]. It is unclear though, as to why these antibodies are generated when a celiac patient eats gluten. One association between tTG and gliadin is that intestinal T cells from celiac patients respond to specific gliadin peptides that have been deamidated, a process that is mediated by tTG binding to gliadin peptides[4]. Anti-tTG IgA is tightly associated with the development of enteropathy and brings into question whether it is a cause or consequence of enteropathy[5,6]. It is especially perplexing because many celiac patients will produce IgA against whole gliadin, a storage protein of gluten, yet this production has a much lower specificity for celiac disease than the anti-tTG IgA ELISA assay[7].

One theory for the origin of anti-tTG IgA was proposed in 1997[8]. This proposal was based on a hapten-carrier model wherein gliadin-specific T cells contribute to the stimulation of B cells that are specific for tTG. This would be achieved by the tTG-specific B cells internalizing complexes of tTG and gliadin peptides and later

presenting gliadin epitopes to the gliadin-specific T cells. In this manner, gliadin-specific T cells would contribute to the tTG-specific B cells producing antibodies against tTG. At that time, this proposal was supported by a lack of evidence for tTG-specific T cells. It is notable that to this date, there is still no evidence that a tTG-specific T cell exists in the small intestine of celiac patients. Of course, it is difficult to prove the absence of a cell type. However, novel ELISAs have been recently developed that can detect antibodies against deamidated gliadin peptides (DGP) in celiac patients[9,10]. This allows us to look at this process in an indirect manner, by determining the correlation among the production of antibody isotypes against DGP and tTG.

MATERIALS AND METHODS

Subjects and study design

Serum samples were collected from patients referred to the division of Gastroenterology and Hepatology at the Mayo Clinic, Rochester, MN, USA for the assessment of gastrointestinal symptoms, unexplained weight loss/anemia, or to rule out celiac disease. One hundred and twenty-one celiac patients were initially included in the study. We defined the diagnosis of celiac disease based on the presence of villous atrophy (enteropathy type ⅢA or greater based on currently accepted diagnostic criteria) in histopathological examination of small intestinal biopsy[11,12]. Of 121 celiac patients who were initially included in the study, 10 were excluded because they had Marsh I enteropathy (n = 8) or IgA deficiency (n = 2). One hundred and ninety-four serum samples were collected from the remaining 111 biopsy-proven celiac patients. Ninety-two samples were collected before patients started treatment and 102 samples were collected while patients were on a gluten-free diet (GFD). The median (range) treatment with GFD was 10.5 (2-54) mo. The study was approved by the Institutional Review Board of Mayo Clinic.

Serology

Anti-DGP IgG and IgA were measured with "QUANTA Lite Gliadin-IgA Ⅱ and Gliadin-IgG Ⅱ" ELISA kits (INOVA Diagnostics Inc., San Diego, CA, USA). Anti-tTG IgA and IgG were measured using "BINDAZYME human IgA and IgG Anti-Tissue Transglutaminase EIA" ELISA kits (The Binding Site, Ltd., Birmingham, UK).

Statistical analysis

Correlations between the antibody titers were assessed by Spearman's rank correlation coefficients that were calculated using version 6.0.0 JMP software (SAS Institute Inc., Cary, NC, USA).

RESULTS

The production of IgA and IgG specific for DGP and tTG was evaluated in celiac patients and plotted such that a direct comparison was made between the production of IgG versus IgA for each antigen group and each patient group (Figure 1). There was a significantly stronger correlation between the production of IgA and IgG specific for DGP (r = 0.75) in celiac patients than those specific for tTG (r = 0.52). When untreated celiac patients (gluten-containing diet; GCD) were separated from treated celiac patients (GFD), the correlation coefficients in comparing anti-DGP IgG and IgA were 0.78 for GCD and 0.58 for GFD, whereas a significantly lower correlation was found for comparing anti-tTG IgG and IgA (r = 0.60 for GCD and r = 0.44 for GFD).

Comparisons were also made between the production of anti-tTG IgA and the production of DGP IgA and IgG in celiac patients (Figure 2). Anti-tTG IgA was highly correlated with the production of both anti-DGP IgA (r = 0.80) and DGP IgG (r = 0.67) which was similar to a previous finding[9].

Finally, comparisons were made between the production of anti-tTG IgG and the production of IgA and IgG specific for DGP. In contrast to anti-tTG IgA which was strongly correlated with DGP antibodies, anti-tTG IgG was weakly correlated with the production of anti-DGP IgA (r = 0.38) and anti-DGP IgG (r = 0.43).

DISCUSSION

The data presented in this manuscript support the theory that the generation of anti-tTG IgA is directly linked to the B cell immune response against DGP, possibly even the T-cell immune response to DGP as well. The reduced correlation in celiac patients between the production of anti-tTG IgG and anti-tTG IgA (r = 0.52) as compared to the production of anti-DGP IgG and anti-DGP IgA (r = 0.75) also demonstrates that there is a fundamental difference between the generation of antibody isotypes against the two antigens in celiac patients. Another difference between the production of IgG and IgA against DGP and tTG is that dietary gliadin mainly affects the production of both IgG and IgA against DGP, but not against both tTG IgG and IgA.

The lack of correlation between the production of anti-tTG IgG and anti-DGP IgG and IgA and anti-tTG IgA (Figures 1 and 2) therefore raises several questions. If the inflammatory T cells that are specific for deamidated gliadin are providing help to the B cells that are specific for a tTG/gliadin complex, why are not more tTG-specific B cells isotype class switching to IgG? One explanation is that the tTG/gliadin-specific B cell group in well-established celiac patients is fully committed to being IgA-positive. A memory B cell could be one such type of long lived IgA-positive B cell, and in this way, require minimal help from a bystander T cell response in order to be fully activated. It is notable that treated celiac patients will relapse with a gluten challenge, even after years of adhering to a GFD, indicating that there is a strong memory component of T cells, B cells, or both in celiac patients[13-15].

Also of interest are studies that demonstrated significant variability in the production of anti-tTG

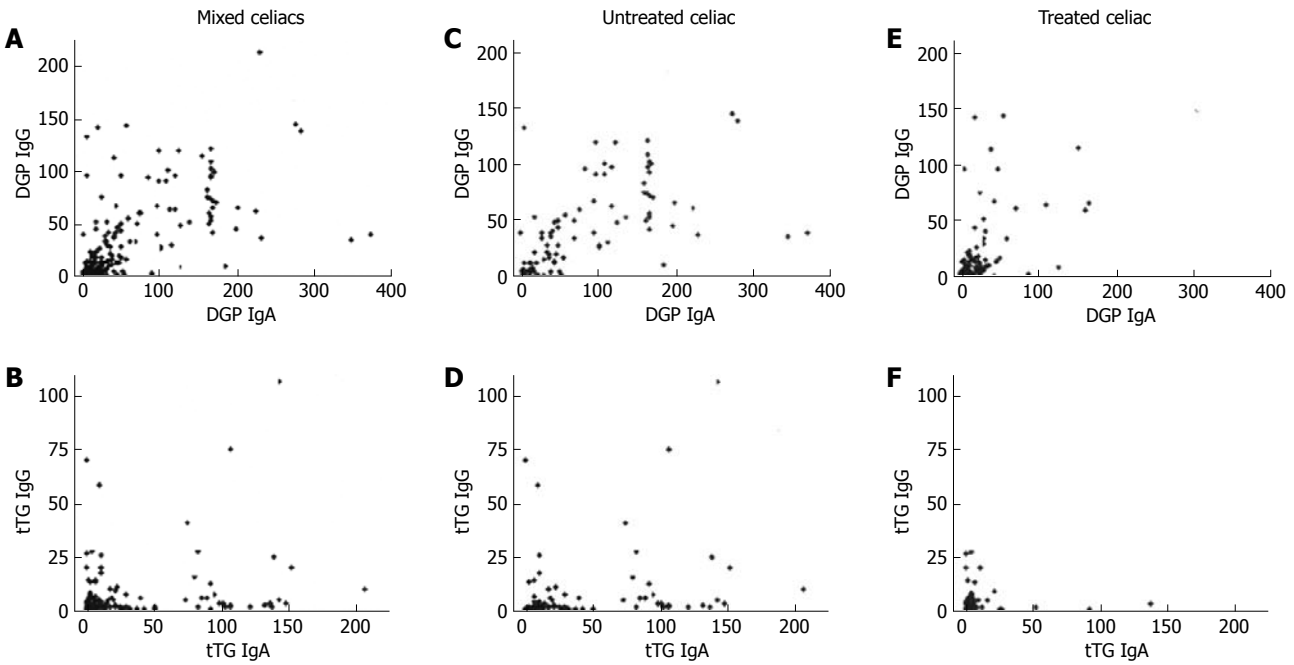

Figure 1 Effect of diet upon isotype correlations. The titers of IgG and IgA against DGP and tTG were evaluated and plotted against each other for celiac patients. For mixed (treated and untreated) celiac patients, the Spearman's rank correlation coefficients were $r = 0.75$ for DGP (A) and $r = 0.52$ for tTG (B). For untreated celiac patients, $r = 0.78$ for DGP (C) and $r = 0.60$ for tTG (D). For treated celiac patients, $r = 0.58$ for DGP (E) and $r = 0.44$ for tTG (F).

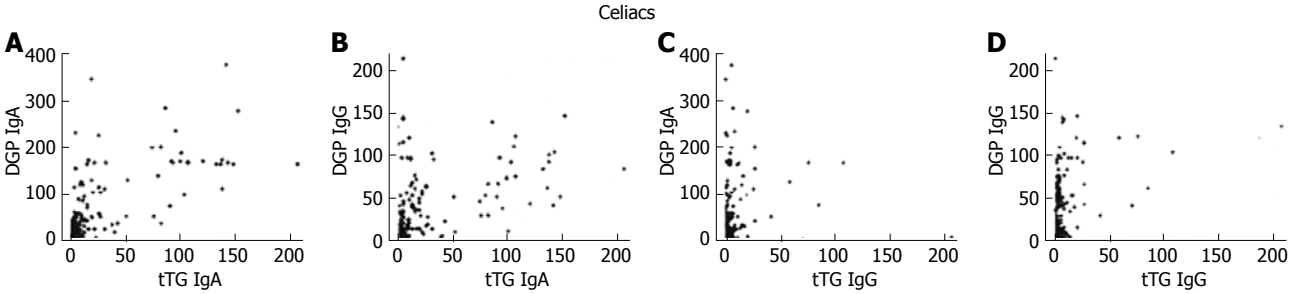

Figure 2 Comparing anti-tTG IgA and IgG production with anti-DGP IgA and IgG. The titers of anti-tTG IgA (A-B) and anti-tTG IgG (C-D) were compared with the titers of anti-DGP IgA (A and C) as well as anti-DGP IgG (B and D) in all treated and untreated celiac patients. Spearman's rank correlation coefficients were 0.80 (A), 0.67 (B), 0.38 (C), and 0.43 (D).

IgA in children. One study found that only six out of 14 celiac patients less than 2 years of age had anti-endomysial antibodies[16]. Another group reported markedly fluctuating levels of transglutaminase antibodies in children[17]. These data would indicate that anti-tTG antibodies do not develop immediately at the time of initial exposure to dietary gliadin, but instead develop after 1-2 (or more) years of continued gliadin exposure.

Our data, as well as the data from others, therefore support the hapten-carrier theory that a B cell that is specific for a tTG/gliadin complex is present in well-established celiac patients and is helped by gliadin-specific T cells. However, our data are also compatible with the model based on molecular mimicry between tTG and gliadin[9,18]. Indeed, it is our belief that the hapten-carrier model and molecular mimicry model are not exclusive. A potential "combined" model would be that a catalyst-like IgA+ memory B cell specific for regions that are shared between tTG and gliadin exists long term in well-established celiac patients. With the consumption of gliadin, these B cells would internalize tTG/gliadin complexes, become activated with minimal T cell help, and then present gliadin peptides to gliadin-specific T cells. This would result in the amplification of both deamidated gliadin specific T- and B-cell responses, as well as the production of anti-tTG IgA antibodies.

COMMENTS

Background

The origin of anti-tissue transglutaminase (tTG) IgA in celiac disease has proven to be elusive and currently two theories exist. One theory is a hapten-carrier model, whereby gliadin-specific T cells provide help for tTG-specific B cells. The other is based on molecular mimicry between tTG and gliadin.

Research frontiers

The recent detection of antibodies in celiac patients specific for deamidated gliadin peptides (DGP), the product of tTG binding to gliadin peptides, provides an opportunity to address the correlation between the production of anti-tTG IgA and the antibodies against DGP in celiac patients.

Innovations and breakthroughs

This study has made the novel observation that the production of both IgG and

IgA against DGP is significantly correlated with the production of anti-tTG IgA and weakly with anti-tTG IgG. This would indicate that the T and B cell response against DGP is fundamentally different from the T- and B-cell response against tTG, and would therefore support the hapten-carrier theory of the origin of tTG IgA.

Applications

By determining the origin of anti-tTG IgA in celiac disease, we obtain a better understanding of the (potentially pathogenic) role of anti-tTG IgA in the development of celiac disease.

Terminology

DGP and tTG are terms that refer to deamidated gliadin peptides and tissue transglutaminase, respectively. Also, gluten free diet (GFD) and GCD refer to gluten-free diet and gluten-containing diet.

Peer review

The data presented in this rapid communication are of interest to the celiac disease community. It is a rapid communication that examines the pattern of serum IgG and IgA levels specific to DGP and tTG in celiac disease patients. It also determines how the administration of a GFD therapy affects this pattern.

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Epidemiology of hepatitis B virus infection in Albania

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Abstract

AIM: To assess the prevalence and socio-demographic distribution of hepatitis B virus (HBV) infection in Albania.

METHODS: Blood samples from 410 unselected schoolboys, 666 students, 500 military personnel, 1286 casual blood donors, 378 voluntary blood donors and 640 pregnant women (total 3880 non-vaccinated residents of rural and metropolitan areas from all over Albania; 2354 (60.7%) male and 1526 (39.3%) female; mean age of 26.3 years) were tested during 2004-2006 for hepatitis B surface antigen (HBsAg) and antibodies to hepatitis B virus (anti-HBs) by ELISA.

RESULTS: The HBsAg and anti-HBs prevalence were 9.5% and 28.7%, respectively. The highest HBsAg prevalence was evident in the younger age group, such as in schoolchildren (11.8%) and the military (10.6%). Consequently, the anti-HBs prevalence increased with age, from 21.2% in schoolchildren (mean age: 15.7 years), to 36.3% in pregnant women (mean age: 26.3 years) and 29.7% in voluntary blood donors (mean age: 40.1 years). There were no significant differences between males and females.

CONCLUSION: Despite the estimated two-fold reduction of HBsAg prevalence in the general population from about 18%-19% to 9.5%, Albania remains a highly endemic country (i.e. over 8% of HBsAg prevalence rate).

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Key words: Albania; Hepatitis B virus; Blood donor; Military; Pregnant women; Schoolchildren; Student

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INTRODUCTION

Hepatitis B is a disease of global distribution. It is estimated that about 30% of the world's population, i.e. approximately 2 billion people, show serological evidence of hepatitis B virus (HBV) infection and about 40 million are persistent carriers of HBV [1]. Each year over one million people die from HBV-related chronic liver disease, including cirrhosis and hepatocellular carcinoma[2].

The endemicity of HBV infection varies greatly worldwide and is influenced primarily by the age at which infection occurs[3,4]. In Europe, the level of endemicity increases from north to south and from west to east. Most countries of northern and western Europe have a very low prevalence of HBV infection (less than 0.5% of the population being positive for HBsAg). Unexpectedly high prevalence of hepatitis B carriage (5%-12%) have been found in many parts of central and Eastern Europe and the former Soviet Union countries[5,6]. Endemicity of infection is considered high in those parts of the world where at least 8% of the population is HBsAg positive. Almost all infections

occur either during the prenatal period or early in a childhood, which accounts for the high rates of chronic HBV infection in these populations[7].

Credible epidemiological data of HBV infection in Albania, before the introduction of obligatory vaccination of newborn children against HBV (1995), was obtained by screening Albanian refugees during the first mass scale migration from Albania to Italy and Greece that occurred in 1991[8-10]. Although the refugees represented mostly subjects from lower socio-economic classes, the large number of people enrolled from different geographic areas (rural and urban) provided important information on HBV infection in Albania (Table 1). The presence of one or more serological markers of HBV infection and the high rate of infection in children aged 1 to 10 years confirms the endemic nature of this virus in Albania.

The above-mentioned data of HBV infection in Albania were undoubtedly related to low hygiene and poor economic situation, overcrowded conditions, lack of disposable needles and syringes, lack of safe blood and its products for transfusion, inadequate sterilization of reusable equipment, difficulties in obtaining appropriate personal equipment to prevent exposure to blood, and lack of an immunization program against HBV.

In 1992, WHO recommended that all countries should include hepatitis B vaccine in their routine infant immunization programs. Since May 1995, thanks to the Rotary International Club, Albania introduced vaccination of newborn children against HBV into the National Immunization Programs as the most appropriate immunization strategy to reduce the rate of HBV infection and HBV-related chronic liver diseases. Infants are immunized at birth, and then after 1 and 5 mo.

MATERIALS AND METHODS

Blood samples from 3880 randomly selected non-vaccinated residents of rural and urban areas from all over Albania were tested during 2004-2006 for HBsAg and anti-HBs by ELISA The blood samples were obtained from 2354 (60.7%) males and 1526 (39.3%) females (mean age of 26.3 years) comprising 410 schoolchildren, 666 students, 500 military, 1286 casual blood donors, 378 voluntary blood donors and 640 pregnant women. Casual blood donors included individuals who donated blood only once, whereas voluntary blood donors included regular blood donors (Table 2). We took blood samples randomly from schoolchildren from several high schools, students from the University of Tirana and soldiers from several military units in main districts of Albania. We also collected blood samples from all casual blood donors and voluntary blood donors during 2004-2005 at the Blood Bank Centre of Tirana. The origin of the subjects was approximately equally distributed between rural and urban regions (1834 rural, 1846 urban).

RESULTS

Baseline characteristics of the study population are presented in Table 2.

Table 1 Prevalence of hepatitis B markers in Albanian refugees according to studies in Italy and Greece

Author	Sanantonio *et al*	Dalekos *et al*	Malamitsi-Puchner *et al*
Study region	Bari	Ioannina	Athens
Yr	1993	1995	1996
Ages	Adults	All ages	Pregnant women
No. cases	393	1025	500
% prevalence of HBsAg	19	22.2	13.4
% prevalence of anti-HBs	55	52	53

Table 2 Baseline characteristics of the study groups

Study groups	Characteristics			
	No (%)	M/F (%)	Mean age (yr)	Yr
Schoolchildren	410 (10.6)	264 (64.4)/ 146 (35.6)	15.7 ± 1.2	2004
Students	666 (17.2)	340 (51.1)/ 326 (48.9)	23.1 ± 1.7	2005
Military	500 (12.9)	500 (100)/ 0 (0)	19.2 ± 2.3	2005
Casual blood donors	1286 (33.1)	987 (76.6)/ 299 (23.3)	32.4 ± 4.8	2004
Voluntary blood donors	378 (9.7)	263 (69.6) 115 (30.3)	40.1 ± 5.1	2005
Pregnant women	640 (16.5)	0 (0) 640 (100)	27.4 ± 4.9	2006
Total	3880	2354 (60.7) 1526 (39.3)	26.3 ± 6.2	2004-2006

Table 3 HBsAg and anti-HBs prevalence in different study groups

Study groups	No. cases	Prevalence (%)	
		HBsAg-positive	antiHBs-positive
Schoolchildren	410	48 (11.8)	87 (21.2)
Students	666	58 (8.7)	247 (37.2)
Military	500	54 (10.6)	124 (24.7)
Casual blood donors	1286	115 (8.9)	293 (22.8)
Voluntary blood donors	378	36 (9.6)	112 (29.7)
Pregnant women	640	47 (7.3)	232 (36.3)
Total	3880	358 (9.5)	1095 (28.7)

The HBsAg and anti-HBs prevalence was 9.5% and 28.7%, respectively. The highest HBsAg prevalence rate was evident in the younger age groups, such as in schoolchildren (11.8%) and in military personnel (10.6%). Consequently, the anti-HBs prevalence increased with age, from 21.2% in schoolchildren (mean age: 15.7 years), to 37.2% in students (mean age: 23.1 years), to 36.3% in pregnant women (mean age: 26.3 years) and 29.7% in voluntary blood donors (mean age: 40.1 years). There were no significant differences between males and females (Table 3). With egard to the age groups, we found prevalence of HBsAg was: 16-20 years: 11.8%; 21-25 years: 9.2%; 26-30 years: 8.3%; 31-35 years: 8.9%; 36-40 years: 9.5%; 41-45 years: 9.5%. We found higher prevalence of HBsAg positivity in urban inhabitants compared with rural inhabitants (11.8% and 7.6%, respectively).

DISCUSSION

The data of this study showed an evident reduction of HBsAg in the general non-vaccinated population of Albania, from 18%-19% (before 1995) to 9.5%. Similar HBsAg prevalence rates were noted among pregnant Albanian women delivering in Greece, and in Albanian health care workers (9.8% and 8.1%, respectively)[11,12].

The success of routine immunization of children and adolescents in interrupting HBV transmission has been previously demonstrated in several high- and low-endemic areas[7]. A primary indicator of the positive impact of hepatitis B vaccination is a reduction of the seroprevalence of HBsAg in the vaccinated population[13]. HBsAg carrier rate in the vaccinated groups has decreased by as much as 74% in less than 10 years in Italy, 96% in 7 years in Saudi Arabia, 93% in 15 years in Taiwan, 79% in 10 years in Thailand, and almost 100% in Alaska[14-18]. Apart from the decreasing seroprevalence of HBsAg in vaccinated populations, another indicator is the decline in the number of acute cases of hepatitis B. Although infections in pediatric age groups are not easy to demonstrate because hepatitis B is rarely symptomatic, trends in the incidence of acute hepatitis B disease can be used to evaluate the influence of vaccination programs in adolescents and adults who are most likely to have asymptomatic infections after HBV exposure[19,13]. In countries such as Italy and the United States, the incidence of acute hepatitis B has declined dramatically during the last decade, particularly among young age groups[20,21]. A significant decline of annual frequency of acute viral hepatitis B from 692 new cases in 2000, to 348 in 2005 was also noted in Albania[22].

Taking into consideration: (1) the reinforcement of the general preventive measures, such as the implementation of the safe injection procedures, proper sterilization of the medical and dental equipment, proper screening of the blood and its products, and progress in health education; and (2) vaccination of some high-risk groups (health care workers, hemodialysis and thalassemic patients), the significant reduction of HBV markers among the non-vaccinated general population (9.5%) compared to the previous rate of 1993-1995 (18%-19%), may be attributed to the 12 consecutive years of vaccination of newborn children against HBV. Similar decreases in HBsAg carrier rates in the non-vaccinated population were also observed in Saudi Arabia and Taiwan[23,24].

The main cause of the reduction in HBsAg prevalence in the general non-vaccinated population (after infant vaccination against HBV) is based on the effective prevention of perinatally transmitted HBV infections among children of HBsAg-positive mothers, and prevention of early childhood transmission between household contacts, which are thought to be responsible for a significant number of HBV infections[18,25-29]. Even in regions with low endemicity, transmission of infection between children and transmission from infected infants to adults has been well documented. This risk of transmission is also demonstrated by the higher infection rate in refugee families and in children's institutions[26]. Furthermore, chronically infected children are likely to be HBeAg positive with a high infectious potential for transmission to other children or adults. Thus, we hypothesize that vaccination programs decrease the risk of HBV infection not only for vaccinated children, but also for all of the population, even those who are non-vaccinated.

COMMENTS

Background

Hepatitis B is a disease of a global distribution. The epidemiological situation of hepatitis B virus (HBV) infection in Albania before the introduction of obligatory vaccination on newborn children against HBV in 1995 was very grave, with high prevalence rates of HBsAg in general population.

Research frontiers

Despite the estimable two-fold reduction of HBsAg prevalence in general population from about 18%-19% to 9.5%, Albania remains a high endemic country.

Innovations and breakthroughs

The vaccination program of newborn children against HBV infection has beneficial effects in the decrease of HBsAg prevalence in non vaccinated population.

Peer review

It is in general well written, organized and interesting.

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Serum biomarker tests are useful in delineating between patients with gastric atrophy and normal, healthy stomach

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Abstract

AIM: To study the value of serum biomarker tests to differentiate between patients with healthy or diseased stomach mucosa: i.e. those with *Helicobacter pylori (H pylori)* gastritis or atrophic gastritis, who have a high risk of gastric cancer or peptic ulcer diseases.

METHODS: Among 162 Japanese outpatients, pepsinogen Ⅰ (Pg Ⅰ) and Ⅱ (Pg Ⅱ) were measured using a conventional Japanese technique, and the European GastroPanel examination (Pg Ⅰ and Pg Ⅱ, gastrin-17 and *H pylori* antibodies). Gastroscopy with gastric biopsies was performed to classify the patients into those with healthy stomach mucosa, *H pylori* non-atrophic gastritis or atrophic gastritis.

RESULTS: Pg Ⅰ and Pg Ⅱ assays with the GastroPanel and the Japanese method showed a highly significant correlation. For methodological reasons, however, serum Pg Ⅰ, but not Pg Ⅱ, was twice as high with the GastroPanel test as with the Japanese test. The biomarker assays revealed that 5% of subjects had advanced atrophic corpus gastritis which was also verified by endoscopic biopsies. GastroPanel examination revealed an additional seven patients who had either advanced atrophic gastritis limited to the antrum or antrum-predominant *H pylori* gastritis. When compared to the endoscopic biopsy findings, the GastroPanel examination classified the patients into groups with "healthy" or "diseased" stomach mucosa with 94% accuracy, 95% sensitivity and 93% specificity.

CONCLUSION: Serum biomarker tests can be used to differentiate between subjects with healthy and diseased gastric mucosa with high accuracy.

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Key words: Gastric atrophy; *Helicobacter pylori*; Serum gastrin-17; Serum pepsinogen

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INTRODUCTION

In 1994, the International Agency on Research for Cancer (IARC) considered *Helicobacter pylori (H pylori)* infection to be a class I carcinogen[1]. *H pylori* infection results in chronic gastritis that will develop into atrophic gastritis of some grade or type in half of infected subjects during their lifetime[2,3]. *H pylori* itself is not carcinogenic but the gastritis it causes, particularly atrophic gastritis, and the subsequent hypochlorhydric

stomach are carcinogenic[1,4-9]. On the other hand, subjects with normal, healthy stomach mucosa have no significant cancer risk, and are also not at risk for peptic ulcer diseases except those who use aspirin or NSAIDs[10]. Therefore, the differentiation between patients with healthy (no *H pylori*, gastritis or atrophic gastritis) and diseased gastric mucosa is clinically relevant. From the viewpoint of cost-effectiveness, this differentiation may be helpful in clinical decision-making and in rationalizing and optimizing diagnostic, therapeutic and screening procedures[11-15].

In the diagnosis of atrophic gastritis, and in the differentiation between healthy and diseased stomach mucosa, two options are available. The first option is gastroscopy and microscopic examination of endoscopic biopsy from the gastric antrum and corpus. The second non-invasive option, is the examination of gastric biomarkers from serum or plasma. Serum levels of pepsinogen (Pg) have been used for decades to diagnose atrophic corpus gastritis non-invasively[16-21]. In particular, in Japan, a country known to have a high prevalence of *H pylori* infection accompanied by gastric atrophy, the usefulness of the serum test to diagnose gastric atrophy has been extensively investigated[22-26], and there has been some success in screening subjects with a high risk of gastric cancer by determining the serum Pg I and Pg I / II ratio[8,27]. Recently, a European biomarker examination, GastroPanel (Biohit Plc, Helsinki, Finland), which not only assays Pg levels but also measures serum or plasma levels of gastrin-17 (G-17) and *H pylori* antibodies (HpAb) of both IgG and IgA class from the same sample using the ELISA technique has been validated[28-30]. In addition to corpus atrophy, the GastroPanel examination also allows exploration of the structure and function of the antrum mucosa, and can indicate the presence of intragastric acidity[31-34].

The aim of this study was to examine, in a Japanese population, how well the European GastroPanel examination delineates patients with atrophic gastritis, and, in particular, how well these examinations differentiate between patients with healthy and diseased gastric mucosa. A second aim was to examine how the conventional Japanese Pg assays fit with those in the European GastroPanel examination.

MATERIALS AND METHODS

Patient series

A total of 162 subjects (95 men) with a mean age of 55 years (range, 22-79 years) who visited the Tohoku University outpatient clinic for upper GI endoscopy were prospectively enrolled in this study from July 2006 to January 2008. The reasons for endoscopic examination were as follow: dyspeptic symptoms in 42 subjects, screening purposes in 54 asymptomatic subjects, annual endoscopic check-up in 38, and positive results during mass screening with barium meal examinations in 28. When enrolling the participants, individuals with a history of gastric surgery, prior *H pylori* eradication therapy, serious systemic disease, and those taking anti-secretory or anti-coagulant drugs were excluded. A fasting blood sample was obtained from each patient before endoscopy, and the serum was separated and stored in a dichotomous fashion at -20℃. An aliquot of each serum sample was subjected to both Pg assay using the Japanese technique and the GastroPanel examination as described below.

Endoscopy and biopsy

Diagnostic upper GI endoscopy was performed in all patients. Endoscopic examination revealed duodenal ulcer scar in 11 subjects, gastric ulcer or gastric ulcer scar in 10, reflux eosophagitis in six, duodenal adenoma in one, gastric adenoma in one, and no abnormal findings or gastritis alone in the others. Endoscopic biopsies were taken from the antrum and corpus, all along the greater curvature (one biopsy from both sites). Biopsy specimens were routinely fixed in neutral formalin and processed in paraffin. Tissue sections were stained with HE, Alcian blue and modified Giemsa (*H pylori* stain) methods.

Classification of patients

Based on histological appearances of the antral and corpus biopsies, the patients were classified into five categories. These categories were:

Atrophic gastritis in corpus alone (C): moderate or severe atrophy (40%-100% loss of normal oxyntic glands with the appearance of intestinal metaplasia and chronic inflammation in varying degree in the available corpus biopsy, in association with normal appearance of the antrum biopsy).

Atrophic gastritis in antrum and corpus (AC): moderate or severe atrophy (40%-100% loss of normal oxyntic and antral (pyloric) glands with the appearance of intestinal metaplasia and chronic inflammation in varying degree in the available antral and corpus biopsies).

Atrophic gastritis in antrum alone (A): moderate or severe atrophy (40%-100% loss of normal antral (pyloric) glands with the appearance of intestinal metaplasia and chronic inflammation in varying degree in the available antral biopsy, in association with normal appearance of the corpus biopsy).

Non-atrophic ("superficial") chronic gastritis (S): no atrophic or metaplastic changes, but the presence of chronic inflammation of varying degree and activity, and with varying grades of *H pylori* in the antrum and/or corpus biopsies.

Normal stomach mucosa (N): mucosa normal in both antrum and corpus biopsy. No atrophy, metaplasia or inflammation.

Categories C, AC and A represented patients with advanced (moderate or severe) atrophic gastritis (AG). Category N represented patients with healthy and normal stomach mucosa. The category of patients with "diseased" gastric mucosa included all those in categories S, A, AC and C.

The biopsy specimens were interpreted by an experienced pathologist (Professor Pentti Sipponen,

Helsinki University Hospital, Helsinki, Finland) without knowledge of the clinical data or results from the biomarker analyses.

Pg Ⅰ and Ⅱ assays with the Japanese technique

Serum levels of Pg were measured by chemiluminescent enzyme immunoassay using commercial kits (Lumipulse pepsinogen Ⅰ & Ⅱ, Fujirebio Inc., Tokyo, Japan)[35]. For the diagnosis of atrophic corpus gastritis, three different criteria were used as follows[22,36,37]: "Mild" criteria: Pg Ⅰ ≤ 70 μg/L and Pg Ⅰ/Ⅱ ≤ 3.0; "Moderate" criteria: Pg Ⅰ ≤ 50 μg/L and Pg Ⅰ/Ⅱ ≤ 3.0; "Strict" criteria: Pg Ⅰ ≤ μg/L and Pg Ⅰ/Ⅱ ≤ 2.0; For each group of criteria, both cut-offs for Pg Ⅰ and Pg Ⅰ/Ⅱ were required to be fulfilled at the same time.

GastroPanel examination

Pg Ⅰ and Ⅱ, amidated gastrin-17, and IgG and IgA class antibodies to *H pylori* were determined using specific ELISA tests (Biohit Plc, Helsinki, Finland) and were performed in batches of 40 samples on a micro-well plate, according to the manufacturer's instructions. All EIA techniques were based on the measurement of absorbance after the peroxidation reaction at 450 nm. Between the reaction steps the plates were washed using a BW50 Microplate Strip Washer (Biohit Plc, Helsinki, Finland). Absorbances were measured using a micro-well plate reader (BP800 Microplate Reader, Biohit Plc, Helsinki, Finland). To determine PgI and gastrin-17 values, second order fits on standard concentrations were used to interpolate/extrapolate from unknown sample concentrations automatically with the help of the BP800 in-built software (Biohit Plc, Helsinki, Finland).

H pylori antibodies were expressed as enzyme immuno units (EIU) according to the formula included in the test kit: Sample EIU = [X (A_{Sample})-X (A_{Blank})]/[X ($A_{Calibrator}$)-X (A_{Blank})]. EIU levels ≥ 30 were considered *H pylori* positive. In the GastroPanel examination, normal ranges for serum/plasma Pg Ⅰ, Pg Ⅱ, Pg Ⅰ/Ⅱ ratio and amidated gastrin-17 were determined by the manufacturer as 30-165 micro/L, 3-15 micro/L, 3-20, and 1-10 pool/L, respectively (www.gastropanel.net).

According to available validations of the GastroPanel examination against endoscopic histology, advanced (moderate or severe) atrophic gastritis was observed with high accuracy ("strict" criteria) if the serum/plasma Pg Ⅰ was < 30 μg/L and/or Pg Ⅰ/Pg Ⅱ ratio < 3[28,29]. Advanced (moderate or severe) antral atrophy or antral predominant *H pylori* gastritis was observed if the HpAb test was positive and fasting serum G-17 < 1 pmol/L.

Classification of patients into different gastritis categories by the GastroPanel examination

Classification of patients using the GastroPanel examination into categories C, AC, A, S or N was carried out using cut-offs for the test parameters as provided by the manufacturer and by using the GastroSoft® computer program (Biohit Plc, Helsinki, Finland). This computer program is based on extensive background material obtained endoscopically and histologically, the program calculates the probabilities for all diagnostic categories from this database. Finally, the GastroSoft program automatically provides the most likely alternative diagnosis. Classification of patients using the GastroPanel examination was done without knowledge of endoscopy and histology results.

Statistical analysis

For the GastroPanel examination and the conventional Japanese Pg assay, the accuracy, sensitivity, and specificity were estimated and compared with histological assessment of the antrum and corpus biopsies. These statistical parameters for the diagnosis of atrophic gastritis were calculated from the serological tests to discriminate histological C, AC, and A from S and N. In the differentiation analysis between patients with healthy and diseased stomach mucosa, these parameters were calculated and used to discriminate C, AC, A, and S from N. The correlations in serum Pg levels between the GastroPanel examination and the conventional Japanese assay were assessed using linear regression analysis, and Pearson correlation coefficients (r) were estimated for each analysis. The study was approved by Tohoku University School of Medicine Ethics Committee and each subject gave written informed consent.

RESULTS

Serum levels of Pg

Serum levels of Pg Ⅰ and Pg Ⅱ correlated significantly and very well ($r = 0.97$, $P < 0.001$ and $r = 0.98$, $P < 0.001$, respectively) between the Japanese assays and the GastroPanel methods in the same serum samples (Figure 1A and B). A technical and methodological difference did exist, however, in that the Pg Ⅰ test in the GastroPanel examination gave exactly twice the Pg Ⅰ level to that in the Japanese assays. No differences were observed between the Pg Ⅱ tests. Accordingly, the Pg Ⅰ/Pg Ⅱ ratio in the GastroPanel examination was exactly twice the ratio in the conventional Japanese tests, even though the correlation between the Pg ratios was highly significant and very good (Figure 1C; $r = 0.96$, $P < 0.001$).

Atrophic gastritis

GastroPanel: Using the "strict" criteria for advanced atrophic gastritis (moderate or severe in grade; see Materials and Methods), Table 1 shows the distribution of patients into the different gastritis categories. When compared to endoscopic histology, the accuracy of the GastroPanel examination to diagnose atrophic gastritis was 87%, the sensitivity was 40% and the specificity 94%.

Conventional Japanese Pg assay: Using the "strict" criteria for cut-offs of Pg levels (Pg Ⅰ ≤ 30 μg/L and Pg Ⅰ/Ⅱ ≤ 2.0) in the Japanese assay, Table 2 shows the distribution of patients into positive and negative groups regarding atrophic gastritis. When compared to endoscopic histology, the accuracy, sensitivity and specificity of the test were 88%, 45% and 96%,

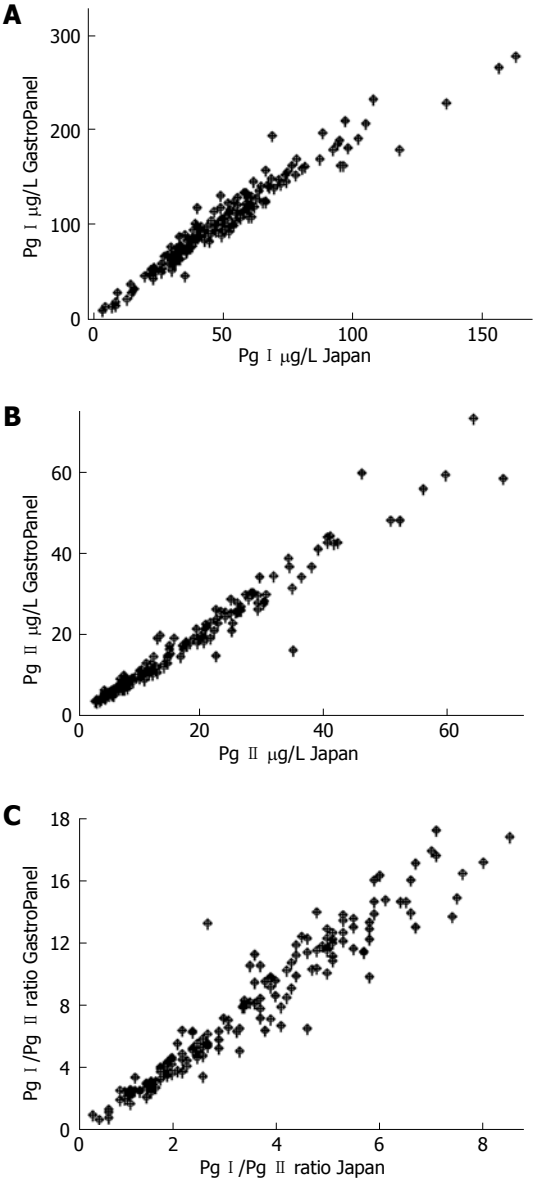

Figure 1 Serum levels of Pg using the GastroPanel examination and the Japanese Pg test in the same serum samples. A: Serum Pg Ⅰ; B: Serum Pg Ⅱ; C: Serum Pg Ⅰ/Ⅱ ratio.

respectively, which corresponded well with those from the GastroPanel examination. If the pepsinogen criteria were lowered to "moderate" (Pg Ⅰ ≤ 50 μg/L and Pg Ⅰ/Ⅱ ≤ 3.0) or "mild" (Pg Ⅰ ≤ 30 μg/L and Pg Ⅰ/Ⅱ ≤ 2.0), the sensitivity of the examination increased but the specificity decreased (Table 3).

Differentiation between patients with healthy and diseased stomach mucosa

The GastroPanel examination, but not Pg testing alone, enabled the differentiation of patients into those with healthy or diseased mucosa (presence of *H pylori* gastritis or atrophic gastritis which may be *H pylori* positive or negative). The GastroPanel test included assays of Pg Ⅰ and Pg Ⅱ as biomarkers for atrophic gastritis in corpus mucosa but also included an amidated G-17 assay as a biomarker of structure and function of the gastric antrum, and assays for the presence or absence of *H pylori* antibodies as a biomarker of on-going *Helicobacter* infection and gastritis. If all biomarkers in the GastroPanel examination were normal, the stomach mucosa was considered normal and healthy. If any of the biomarkers were abnormal, the patient was considered to have *H pylori* gastritis or atrophic gastritis. Using this delineation (see Materials and Methods), Table 4 shows the distribution of the patients into two subgroups (i.e. those with healthy and normal stomach versus those with *H pylori* gastritis or atrophic gastritis). In this setting, the findings from biopsy histology were compared between the two delineated subgroups. In this analysis, the accuracy, sensitivity and specificity of the GastroPanel test to diagnose healthy stomach mucosa were 94%, 95% and 93%, respectively.

Table 1 Prevalence of patients in different gastritis categories. Comparison between GastroPanel examination and biopsy histology

GastroPanel	Histology					
	C	AC	A	S	N	Total
C	3	0	0	4	0	7
AC	1	2	0	0	0	3
A	0	1	1	5	0	7
S	3	1	5	73	3	85
N	0	2	1	4	53	60
Total	7	6	7	86	56	162

Accuracy: 87%; sensitivity: 40%; specificity: 94%; C, AC, A: Moderate or severe atrophic gastritis in corpus alone, in antrum and corpus simultaneously, and in antrum alone, respectively; S: Non-atrophic *H pylori* gastritis; N: Normal and healthy stomach mucosa.

Table 2 Prevalence of patients in corpus atrophy positive (AG+) and negative (AG-) categories if "strict" criteria for cut-off of positive Pg test (Pg test+ versus Pg test-) are used. Comparison between Japanese Pepsinogen test and biopsy histology

	AG+	AG-	Total
Pg test+	9	6	15
Pg test-	11	136	147
Total	20	142	162

AG: Atrophic corpus gastritis present (+) or absent (-). Pg: Pepsinogen test positive (+) or negative (-) for atrophic corpus gastritis.

Table 3 Sensitivity and specificity of the Japanese pepsinogen test and GastroPanel examination in atrophic gastritis if the cut-offs (criteria) for the positive pepsinogen test result are set to be mild, moderate or strict (%)

Pepsinogen test criteria	Sensitivity	Specificity
Japanese-mild	75	69
Japanese-moderate	65	77
Japanese-strict	45	96
Gastropanel-strict	40	94

DISCUSSION

The present analysis showed that non-invasive serum Pg assays accurately diagnosed Japanese patients with atrophic corpus gastritis. Similar findings were also obtained

Table 4 Prevalence of patients in categories of "healthy" or "diseased" gastric mucosa. Comparison between GastroPanel examination and biopsy histology

GastroPanel	Histology		
	Healthy stomach mucosa	Diseased stomach mucosa	Total
Healthy stomach mucosa	53	7	60
Diseased stomach mucosa	3	99	102
Total	56	106	162

Accuracy: 94%; sensitivity: 95%; specificity: 93%.

using both the conventional Japanese Pg tests and the Pg assays of the novel European GastroPanel examination in which, in addition to Pg, the serum/plasma levels of amidated gastrin-17 (G-17) and *H pylori* antibodies (IgG and IgA) were also measured. The diagnostic accuracy of both the Japanese test and the GastroPanel test was more than 80% when compared with endoscopic biopsy histology. In addition, it is noteworthy that, since both the Japanese and the European (GastroPanel) Pg tests seemed to fit without any exceptions, no racial differences could be demonstrated in Pg antigens by the present study-both the Japanese and European assays gave very similar results.

The GastroPanel test included assays of amidated G-17 and *H pylori* antibodies in addition to the Pg assays. The rationale for this is that the serum level of amidated G-17 is a biomarker of the function and structure of the gastric antral mucosa. Serum levels of G-17 were high in subjects with atrophic gastritis limited to corpus mucosa alone but normal and low in those in whom atrophic gastritis was present in both the antrum and corpus (multifocal atrophic gastritis of Correa - highest risk condition for gastric cancer known so far). The rationale for the serological *H pylori* test, on the other hand, is that the presence or absence of *H pylori* antibodies in serum is the most reliable biomarker of an on-going *H pylori* infection. When compared with the ^{13}C urea breath-test (UBT) or stool antigen test, the serological test avoids false-negative results which appear in more than half of patients with atrophic corpus gastritis (hypochlorhydric stomach) or PPI use when analyzed using the UBT or stool antigen test. In this sense, the GastroPanel biomarker examination provides a most reliable tool for delineating between patients with healthy stomach and those with *H pylori* non-atrophic gastritis or atrophic gastritis.

In the present study, the biomarker tests were compared with endoscopic biopsy histology. Endoscopic biopsy histology is, however, not a reliable gold standard. Biopsy results are commonly biased by several factors, including such confounders as biopsy sampling, number of biopsies available from each stomach compartment, laboratory processing of the specimens, and interpretation of the biopsy by pathologists. In the present study, the biopsy analysis was based on only one biopsy from both the antrum and corpus, and so the study protocol did not strictly follow the guidelines of the Sydney System (the guidelines indicate at least two biopsies from each compartment). Interpretation of the biopsy findings by pathologists may, therefore, easily fail, particularly in antral biopsies, in which the interobserver agreement, even between "expert" pathologists, is known to be imperfect and may require practice or even the application of morphometry[38-40].

Biomarker examinations from serum or plasma are free of the biases that affect biopsy histology or sampling. The biomarkers give an average view of the structure and function of the stomach mucosa. In addition to the Pg tests, the GastroPanel examination included assays of serum/plasma levels of amidated G-17 and *H pylori* antibodies. This also allows insight into the function and structure of the gastric antrum, confirms Pg assays, and can suggest the presence of intragastric acidity[31-34]. A low fasting level of serum/plasma G-17 indicates subjects with high intragastric acidity (acid inhibits the release of amidated G-17 from antral G cells) or those with atrophy of the antral mucosa (the loss of antral glands also results in loss and disappearance of antral G cells)[28,29]. In the present study population, seven patients were classified into this category according to the GastroPanel examination. These seven patients were anticipated to have an antrum-limited atrophic gastritis or *H pylori* gastritis that was strongly antrum predominant (a phenotype of *H pylori* gastritis that is associated with the risk of peptic ulcer disease (PU), particularly PU of the duodenal ulcer type)[10]. Low fasting levels of serum/plasma G-17 in connection with low Pg I or Pg I /Pg Ⅱ ratio also identifies subjects who have the highest known risk of gastric cancer; i.e. patients with advanced and extensive atrophic gastritis in both the antrum and corpus (advanced multifocal atrophic gastritis)[4,6,17]. In the present study, three patients (2%) were classified into this category using the GastroPanel examination, which was also confirmed by biopsy histology.

Differentiation between patients with healthy and diseased gastric mucosa is one of the key issues in assessing the risks for serious gastric diseases in clinical practice. If the stomach mucosa is healthy, the risks of serious gastric diseases (cancer or peptic ulcer) are extremely low (nil in practice). With high certainty (accuracy 94%, sensitivity 95% and specificity 93%) the GastroPanel examination indicated that 53 of 162 patients (33%) in this study had normal and healthy stomach mucosa.

Biomarker tests are not "cancer tests". However, they can be used in the screening and diagnosis of subjects with a high cancer risk; i.e. subjects with atrophic gastritis in which a careful diagnostic endoscopy (gastroscopy) is mandatory to find possible neoplastic or precancerous lesions at an early and curable stage. In the post hoc analysis, none of these 53 patients with "healthy" stomach had neoplastic lesions or signs of active peptic ulcers on endoscopy (one patient had a duodenal scar and one had a scar in the stomach mucosa). On the other hand, two of the patients with atrophic gastritis had neoplastic gastric or duodenal adenoma. Thus, in the present study population, all neoplastic gastroduodenal

lesions were found in those patients with diseased stomach mucosa using the GastroPanel examination.

The reasons for the differences in serum levels of Pg I between the Japanese and GastroPanel assays are technical and methodological, and are most likely due to differences in the calibrators used in the assay technique. However, due to the excellent correlations between the tests, the results from the conventional Japanese Pg I assay can easily be converted (by doubling the test results) to correspond with those obtained using the GastroPanel Pg I test, or *vice versa*.

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COMMENTS

Background

Reliable non-invasive diagnosis of *Helicobacter pylori (H pylori)* gastritis and atrophic gastritis, and the delineation of patients with healthy stomach mucosa, are clinically important tasks.

Research frontiers

Biomarker tests are potential non-invasive diagnostic tools for assessment of the function and structure of the stomach mucosa.

Innovations and breakthroughs

Available pepsinogen (Pg) tests, both Japanese and European, are excellent in Asian outpatients when compared in a "head-to-head" analysis in the same study population. The addition of assays for serum amidated gastrin-17 and serological *H pylori* tests to the Pg assays increases the clinical applicability of the biomarker tests.

Applications

A comprehensive set of biomarker tests (GastroPanel) is applicable in the reliable diagnosis of *H pylori* gastritis, atrophic gastritis, and also in the delineation of subjects with healthy, normal stomach mucosa.

Peer review

The authors evaluated the predictive value of the detection of a set of serum biomarkers (Pg Ⅰ/Pg Ⅱ, gastrin-17, and antibodies against *H pylori*) using the European GastroPanel examination among 162 Japanese patients. They found that the GastroPanel examination classified the patients into groups with "healthy" or "diseased" stomach mucosa with 94% accuracy, 95% sensitivity and 93% specificity, as compared to endoscopic biopsy findings. It is helpful for readers to understand the usefulness of this examination among Asian gastric patients.

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BRIEF ARTICLES

Furazolidone, amoxicillin, bismuth and rabeprazole quadruple rescue therapy for the eradication of *Helicobacter pylori*

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Abstract

AIM: To compare the efficacy and side effect profiles of three furazolidone and amoxicillin-based quadruple rescue therapies for the eradication of *Helicobacter pylori* (*H pylori*).

METHODS: Patients who failed in the *H pylori* eradication therapy for at least one course were randomly allocated into three groups. Group A received rebaprazole 10 mg + amoxicillin 1 g + furazolidone 100 mg, and bismuth subcitrate 220 mg, twice daily for 1 wk; group B received the same regimen of group A but for 2 wk; and group C received the same regimen of group B, but furazolidone was replaced by furazolidone 100 mg three times daily. To record the side effect profiles at the end of the treatment, *H pylori* eradication was assessed with ^{13}C-urea breath test 4 wk after therapy.

RESULTS: Sixty patients were enrolled including 28 males, and 20 patients in each group. The average age of the patients was 49.2 years, ranging from 18 to 84 years. *H pylori* eradication rates with per-protocol analysis were 82%, 89% and 90% in the three groups, respectively. Side effects were found in 11 patients, including mild dizziness, nausea, diarrhea and increased bowel movement. None of the 11 patients needed treatment for their side effects.

CONCLUSION: One- or two-week furazolidone and amoxicillin-based quadruple rescue therapy with a low dose furazolidone (100 mg *bid*) for the eradication of *H pylori* is effective. Extending the antibiotic course to 14 d could improve the eradication rates.

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Key words: *Helicobacter pylori*; Rescue therapy; Quadruple therapy; Furazolidone; Low dose

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INTRODUCTION

Helicobacter pylori (*H pylori*) infection is associated with many upper gastrointestinal diseases, such as chronic gastritis, peptic ulcer, gastric carcinoma and mild malignant mucosa-associated lymphoid tissue lymphoma (MALToma). During recent years, the efficacy of the first-line therapy including proton pump inhibitors plus two antibiotics seems to have decreased, and several studies have reported intention-to-treat eradication rates lower than 75%[1-3] and even lower than 50%[4,5]. The resistance to antibiotics is the main cause of *H pylori* treatment failure. A multicenter study conducted in China in 2005 showed that the resistant rates of *H pylori* were 27.4% for clarithromycin, 75.6% for metronidazole and 2.7% for amoxicillin[6]. Many patients needed to receive rescue therapy for the eradication of *H pylori* after first- or second-line therapies. The combination of metronidazole, tetracycline, bismuth, and proton pump inhibitor are currently considered standard rescue regimens for the treatment of *H pylori* infection[7]. However, metronidazole resistance is a rising problem worldwide, particularly in developing countries, such as China, which limits the usefulness of this drug.

Furazolidone, a monoamine oxidase inhibitor, has broad antibacterial activity based on interference with bacterial enzymes. It has already been used to treat peptic ulcer disease for many years in China, before *H pylori* was discovered[8,9]. Furazolidone emerged as an agent for *H pylori* eradication regimens due to its low cost and prevalence of resistant strains in China.

The consensus of reports of China in 2005[10] and 2007[11] all recommended that furazolidone should be used for *H pylori* eradication treatment. A large multicentrer study in China showed that a combination of omeprazole plus furazolidone and amoxicillin as the first-line regimen had an intention-to-treat *H pylori* eradication rate of 86%, compared to 69% for omeprazole plus clarithromycin and metronidazole[12]. However, in another study in China, the intention-to-treat eradication rate of omeprazole-furazolidone-amoxicillin regimen as the rescue regimen was only 52%[13]. The aim of this pilot study was to compare the efficacy and side effect profiles of three different furazolidone and amoxicillin-based quadruple rescue therapies for the eradication of *H pylori*.

MATERIALS AND METHODS

Subjects

This prospective clinical trial was conducted in Peking University First Hospital. Patients who failed in the eradication of *H pylori* for at least one course were invited to participate in this open-label pilot study. Informed written consent was obtained from all patients participating in the trial.

Patients younger than 18 years of age, and who presented with severe comorbidity, who were pregnant or lactating, with a known history of allergy to the study drugs, and patients who had used proton pump inhibitors, H_2 receptor blockers, antibiotics, or bismuth salts up to 4 wk before the study were all excluded.

Treatment regimen

Patients were randomly allocated into three groups. Group A received rebaprazole 10 mg, amoxicillin 1 g, furazolidone 100 mg, and bismuth subcitrate 220 mg, twice daily each, for 1 wk; group B received the same regimen as group A but for 2 wk; and group C received the same regimen of group B but furazolidone was replaced by furazolidone 100 mg three times daily. Antibiotics were prescribed after meals whereas rabeprazole and bismuth were adminstered before meals. Patients were advised to maintain the treatment even with minor adverse effects. No other medication was allowed until the end of the treatment.

Assessment

Patients were evaluated using the ^{13}C-urea breath test at least 4 wk after *H pylori* eradication treatment. Antimicrobials, bismuth-containing drugs and acid-reducing agents were not allowed during the 4 wk preceding the ^{13}C-urea breath test. The eradication of *H pylori* was defined as a negative urea breath test.

The patient compliance and treatment-related side effects were assessed at the end of the treatment. Side effects were graded as mild if they did not interfere with daily activities of the patients, moderate if they interfered with daily activities to some extent and severe if daily activities became impossible.

Statistical analysis

Continuous variables were expressed by calculation of the mean and standard deviation. The *H pylori* eradication rate was assessed based on intention-to-treat and per-protocol analysis. The 95% confidence intervals (95% CI) were also calculated for both intention-to-treat and per protocol analysis and the eradication rate. The patients, who were lost to follow-up or could not complete the treatment course because of severe side effects, were considered as treatment failures and excluded in the per-protocol analysis. The Chi-square test and Fisher's exact test were used to compare the differences between the three study groups in terms of baseline data, eradication rate and side effects. $P < 0.05$ was considered significant.

RESULTS

Sixty patients were enrolled in this study including 28 males, with 20 patients in each group. All of the patients had undergone endoscopy examination before they received *H pylori* eradication at the first time. The average age of the patients was 49.1 years, ranging from 18 to 84 years. Two patients had already undergone three treatments, 42 and 16 had undergone one and two, respectively. There was no predominance regarding the baseline characteristics of the patients (Table 1).

All the patients finished the treatment, but four of them did not receive the ^{13}C-urea breath test examination. *H pylori* eradication rates with per-protocol analyses were 82%, 89% and 90% in the three groups, respectively ($P > 0.05$) and the intention-to-treat eradication rates were 70% (14/20), 85% (17/20) and 90% (18/20) in the three groups ($P > 0.05$) (Table 2). No significant difference was found between the eradication rates of the patients who failed *H pylori* eradication for one, two or three courses (Table 3).

Side effects were found in 11 patients, including mild dizziness, nausea, diarrhea and bowel movement increase (Table 4). None of the 11 patients needed treatment or stopped the therapy for their side effects.

DISCUSSION

The eradication of *H pylori* is the main objective in the treatment of peptic ulcer[14,15]. The Masstricht Ⅲ consensus report concluded that eradication of *H pylori* has the potential to reduce the risk of gastric cancer development; moreover, the optimal time to eradicate *H pylori* is before pre-neoplastic lesions (atrophy and intestinal metaplasia) are present[7]. Gastric carcinoma is common in China. So *H pylori* infection is a major public health problem, for which treatment should be provided when patients are diagnosed. The ideal therapy for *H pylori* infection should achieve a high cure rate of > 90% on per protocol analysis and > 80% on intention-to-treat analysis, should be simple and well tolerated, and should be easy to comply with and cost-effective[16]. The combination of proton pump inhibitor plus bismuth, tetracycline and metronidazole has been

Table 1 Baseline characteristics of patients (mean ± SD)

Group	No. of patients	Age (yr)	Male	Gastritis	Ulcer (DU/GU)	Smoking	Drinking
A	20	49.3 ± 15.2	8	11	8 (7/1)	6	3
B	20	49.9 ± 21.5	12	13	9 (8/1)	6	5
C	20	48.1 ± 9.3	8	12	7 (7/0)	6	3
Total	60	49.1 ± 15.8	28	36	24	18	9
χ^2 value	-	-	2.14	0.42	0.42	0.00	0.89
P value	-	-	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

Table 2 *H pylori* eradication rates among three treatment groups

Group	Eradication	Non-eradication	Lost	Total	Eradication rate[a] (PP, %) (95% CI, %)	Eradication rate[b] (ITT, %) (95% CI, %)
A	14	3	3	20	82 (57-96)	70 (46-88)
B	17	2	1	20	89 (67-99)	85 (62-97)
C	18	2	0	20	90 (68-99)	90 (68-99)
Total	48	7	4	60	87 (78-96)	80 (70-90)

[a]$\chi^2 = 0.59$, $P > 0.05$; [b]$\chi^2 = 2.89$, $P > 0.05$.

Table 3 *H pylori* eradication rates in relation to course of previous treatment

Times of failure	Eradication	Non-eradication	Lost	Total	Eradication rate[a] (PP,%) (95% CI, %)	Eradication rate[b] (ITT, %) (95% CI, %)
1	35	5	2	42	88 (73-96)	83 (74-96)
2	13	1	2	16	93 (66-100)	81 (54-96)
3	1	1	0	2	50 (1-99)	50 (1-99)
Total	48	7	4	60	87 (78-96)	80 (70-90)

[a]$\chi^2 = 2.94$, $P > 0.05$; [b]$\chi^2 = 1.42$, $P > 0.05$.

Table 4 Side-effect profile of patients *n* (%)

Side effects	Group A	Group B	Group C	Total
Nausea	2	1	2	5 (8.3)
Diarrhea	1	1	1	3 (5.0)
Bowel movement increasing	0	1	1	2 (3.3)
Dizziness	0	0	2	2 (3.3)
Headache	0	0	1	1 (1.7)
Asthenia	1	0	0	1 (1.7)
Total episodes	4	3	7	14 (23.3)
Total patients[1]	2 (10)	3 (15)	6 (30)	11 (18.3)

Two patients got two kinds of side effects in group A and group C, respectively; [1]$\chi^2 = 2.89$, $P > 0.05$.

recommended as optimal second-line therapy by several guidelines on the management of *H pylori* infection[7]. However, metronidazole resistance is largely responsible for treatment failure. The prevalence of metronidazole resistance for *H pylori* is about 80% in China[6]. The best rescue treatment remains to be defined.

Furazolidone is a broad-spectrum nitrofuran, active against Gram-negative and positive bacteria and protozoa by inhibiting bacterial enzymes, and it has poor oral absorption[17]. Strains resistant to furazolidone are rare and have no cross-resistance to metronidazole[18]. Furthermore, its potential to develop resistance is low[19]. Several studies have shown the efficacy of regimens containing a high-dose furazolidone (200 mg, *b.i.d.*) as the therapy in patients with *H pylori* infection[20-22]. The study of Fakheri *et al* showed that low-dose furazolidone (100 mg, *b.i.d.*) based triple and quadruple rescue regimens do not yield acceptable success rates[23]. We have reported that a pilot study of rabeprazole, bismuth, furazolidone and amoxicillin as rescue treatment of *H pylori* infection after failure for at least one course of eradication has intention-to-treat and per-protocol eradication rates of 70% and 82% for 1 wk in the group treated with furazolidone (100 mg, *b.i.d.*), 85% and 89% for 2 wk in the group with furazolidone (100 mg, *b.i.d.*), and 90% and 90% for 2 wk in the group with furozolidone (100 mg, *t.i.d.*), respectively. Our study is different from Fakheri *et al*, as a regimen which is useful in one area may not be effective in another area.

Management of first- or second-line *H pylori* eradication failures has become a challenge. In one study, a *H pylori* eradication rate of 69% was obtained after treatment with a 7-d association of bismuth, high-dose furazolidone (200 mg, *b.i.d.*), amoxicillin and a

proton-pump inhibitor for patients with peptic ulcer who failed to respond to other eradication regimens[24]. In another study, a similar eradication rate (63%, intention-to-treat) was achieved with a rescue treatment of a 7-d quadruple regimen with omeprazole, bismuth, tetracycline, and high-dose furazolidone (200 mg, *b.i.d.*)[25]. Currently, a standard third-line therapy is lacking, and several guidelines recommend a culture to select proper treatment according to microbial sensitivity to antibiotics for these patients[7,11]. However, cultures are often carried out only in research centers. It is a common practice to select a rescue therapy according to experience, especially in China. In this study, the intention-to-treat eradication rates were 73%, 81% and 50% for the patients who failed in *H pylori* eradication for one, two or three courses, respectively. Although, without information of microbial sensitivity to antibiotics, it has been shown that furazolidone-(100 mg, *b.i.d.* or *t.i.d.*) and amoxicillin-based quadruple rescue therapy was highly effective in the population of this region. *H pylori* eradication can be achieved in most patients, even when antibiotic susceptibility is not tested.

Furazolidone presents some side effects, especially gastrointestinal ones[17]. Several studies showed that side effects were very common (more than 20%) in the patients who received treatment with furazolidone-based regimens[13], especially with high-dose furazolidone of 200 mg *b.i.d.*[24,26]. A major problem with furazolidone at high doses is the high rate of severe side effects. Most of these effects are related to its role as a monoamino-oxidase inhibitor and include fever, rash and severe abdominal pain. Such side effects may lead to the discontinuation of treatment in some patients[21]. In this study, the occurrence of side events was 18.3%, and no intolerable side effects leading to early discontinuation of treatment were found. The most common side effects were nausea and diarrhea. Although the side effects were more frequent after extending the treatment course and adding furazolidone, this difference was not significant among different treatment groups.

The weakness of this study is that although set up as a controlled trial, the number of patients in each arm was small. Further studies should be done to conclude whether the increased dose of furazolidone or the longer period of treatment is helpful.

In conclusion, our study shows that the association of rabeprazole, bismuth, amoxicillin, and low-dose furazolidone is a valuable rescue treatment for patients who failed to respond to the first- or second-line *H pylori* eradication in China. Lower doses of furazolidone could decrease the incidence of side effects, but this strategy can also lead to a lower eradication rate. However, extending the antibiotic course to 14 d could improve eradication rates, despite a greater likelihood of side effects. The regimens are well tolerated by most patients. These are effective, cheap and safe options for salvage therapy of *H pylori* positive patients, and may be recommended as good alternative choice regimens in the eradication of *H pylori* in the population with high metronidazole resistance.

COMMENTS

Background

Helicobacter pylori (*H pylori*) infection is associated with many upper gastrointestinal diseases. The prevalence of *H pylori* resistant to antibiotics was increased with the spreading of *H pylori* eradication. Many patients needed to receive rescue therapy for the eradication of *H pylori* after first- or second-line therapies.

Research frontiers

Furazolidone-based regimens for the eradication of *H pylori* are low in cost. Lower doses of furazolidone could decrease the incidence of side effects. Not many studies have been performed to evaluate the efficacy of low-dose furazolidone-based quadruple regimens for treatment of *H pylori* infection.

Innovations and breakthroughs

This study provides further evidence of the efficacy and tolerability of low-dose furazolidone-based quadruple regimens in China.

Applications

Low-dose furazolidone-based quadruple regimens may be useful rescue therapies for *H pylori* eradication due to their low cost, low resistance rate and relatively minor side effects, especially in developing countries such as China.

Peer review

Furazolidone has been used for the eradication of *H pylori* for many years, but here it has been used as a component of quadruple rescue therapy. This has been reported less frequently. In this series of three groups of 20 patients, in which *H pylori* eradication therapy had failed to respond to at least one previous treatment regimen, each group was treated with one of three regimens of quadruple therapy containing furazolidone (either in different doses or for a different time), and the results were highly successful. Side effects from furazolidone are the main disadvantage of this drug, but when used in small doses as in this study, side effects were relatively minor. For the above reasons this is a useful publication.

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Diagnostic model of saliva protein finger print analysis of patients with gastric cancer

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Abstract

AIM: To explore the method for early diagnosis of gastric cancer by screening the expression spectrum of saliva protein in gastric cancer patients using mass spectrometry for proteomics.

METHODS: Proportional peptide mass fingerprints were obtained by analysis based on proteomics matrix-assisted laser desorption ionization time-of-flight/mass spectrometry. A diagnosis model was established using weak cation exchange magnetic beads to test saliva specimens from gastric cancer patients and healthy subjects.

RESULTS: Significant differences were observed in the mass to charge ratio (m/z) peaks of four proteins (1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da) between gastric cancer patients and healthy subjects.

CONCLUSION: The finger print mass spectrum of saliva protein in patients with gastric cancer can be established using gastric cancer proteomics. A diagnostic model for distinguishing protein expression mass spectra of gastric cancer from non-gastric-cancer saliva can be established according to the different expression of proteins 1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da. The method for early diagnosis of gastric cancer is of certain value for screening special biological markers.

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Key words: Saliva; Protein finger print model; Gastric cancer; Matrix-assisted laser desorption ionization-time-of-flight/mass spectrometry; Weak cation exchange; Magnetic bead; Proteomics

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INTRODUCTION

Gastric cancer is one of the most common cancers. Even though its mortality rate has decreased in recent years, its morbidity still ranks first among all kinds of malignant tumor. Moreover, gastric cancer obviously occurs in juvenescence, and is diagnosed at its advanced stage. About 150-200 thousand patients die of gastric cancer every year in our country, which accounts for almost a quarter of all deaths from malignant tumors. About 200 000 new gastric cancer patients are diagnosed each year. At present, available tumor markers have a relatively high false-positive rate for the diagnosis of gastric cancer, and thus cannot predict early gastric cancer[1,2]. Therefore, it has become important to find a special way to predict early stage gastric cancer.

Saliva can be used in diagnosing gastric cancer, and it has been verified for many years that detection of salivary components is a valuable tool to diagnose a variety of diseases[3]. Salivaas, a diagnostic specimen has received attention. Along with the wide application of proteomics and its related techniques, proteomics has been used more widely in the study of saliva[4,5]. In this study, we employed matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) technique to analyze the protein mass peak of

saliva from gastric cancer patients and healthy subjects, and to screen for the special biological markers for predicting early gastric cancer.

MATERIALS AND METHODS

Clinical data and specimen selection

Saliva samples were collected from gastric cancer patients in the Medical Department of Nanfang Hospital, Southern Medical University. Control samples were collected from healthy volunteers. Saliva was collected and put into a 15-mL centrifuge tube, and centrifuged at 10000 r/min for 5 min at 4℃. Fifty microliters of each saliva sample was put into in a 1-mL EP tube and stored at -80℃. When experiments were conducted, the samples were taken out from the refrigerator at -80℃. All samples were defrosted at room temperature. The healthy subjects at the age of 23-68 years served as a normal group (n = 18, 12 males, 6 females) and the patients at the age of 25-78 years served as a gastric cancer group (n = 23, 15 males, 8 females).

Instruments and reagents

A weak cation exchange (WCX) magnetic bead kit, alpha-cyano-4-hvdroxycinnamic acid (HCCA), and AutoFlex III MALDI-TOF mass spectrometer were purchased from Bruker Company. Mass concentration (0.3 g/L) and ethanol (chromatographic grade)/acetone (chromatographic grade) = 2/1 were freshly prepared.

Treatment with WCX magnetic beads

The WCX magnetic bead kit was taken out from a refrigerator at 4℃, washed and eluted. Finally, the separated magnetic beads and eluted polypeptide samples were transferred into a 0.5-mL clean sample tube for further MS analysis.

Sample application and MS analysis

One microliter polypeptide sample separated with the magnetic beads was applied. After the polypeptide sample was dried at room temperature, into which 1 μL HCCA substrate solution (3 g/L, dissolved in 50% acetonitrile and 2% trifluoroacetic acid) was added. Then, the prepared sample was applied and analyzed on MALDI-TOF-MS. A linear mode was used to collect peptides with a molecular weight of 1000-10000. Twenty percent of laser energy was used with 400 shots. Peptide mass finger prints were obtained by accumulating 50 single scanning of MS signals.

Statistic analysis

FlexAnalysis 3.0 and ClinProTools 2.1 (from Bruker Company) were used to analyze the grouping index and its dependability of data. $P < 0.05$ was considered statistically significant.

RESULTS

The mass spectrum of samples from 41 subjects was analyzed and compared. Seventy-four protein mass peaks were found. Mass peaks were proved to be significantly different in four proteins (1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da) (Figures 1-4). The protein mass peak at 1472.78 Da was higher in the gastric cancer group than in the normal group. Based on the identification of these four protein mass peaks, 22 patients were accurately diagnosed with gastric cancer. All the 18 healthy subjects were confirmed to have no gastric cancer. The present method for the diagnosis of gastric cancer had a sensitivity of 95.65% (22/23), and a specificity of 100% (18/18). The results are shown in Table 1.

Table 1 Identification of gastric cancer in different groups

Groups	Classification of proteome mass spectrum model			
	n	Gastric cancer	Non-gastric cancer	Ratio (%)
Gastric cancer group	23	22	1	95.65
Normal group	18	0	18	100

DISCUSSION

Saliva is an important and necessary body fluid. Blood constituents such as hormones, amino acids, electrolyte, immunoglobulin and creatinine, can enter saliva through the blood barriers of the capillary walls. As one of the fluids in the human body, variation in saliva constituents is influenced by various pathophysiological changes in the body. Saliva constituents are related to serum and the essential proteins in saliva are positively correlated with serum. For example, there is an extremely good dependability between blood plasma free F and salvia F, which is not influenced by the flow rate and stimulation of saliva. Many proteins in saliva, such as anti-HIV antibody, secretory type leukoprotease inhibitor (SLPI) and IgA, have important physiological functions. SLPI, a kind of single strand polypeptide, can be separated from human parotid gland saliva and only has anti-HIV effects in the oral cavity. Saliva can be atraumatically and conveniently taken, and easily observed at any time. The test results are stable and the sensitivity is rather high, and the tests can be repeated. Since biochemical microanalysis techniques have been significantly improved, saliva can be used as a diagnostic index instead of blood[3,6-9].

Studies on saliva proteomics have received extensive attention worldwide[4,5]. Till now, 309 kinds of protein have been identified in full salivary fluid of healthy subjects, using proteomics techniques, among which, the most important acidic proteins are catheptic enzyme L and hyaluronan-conjugated protein; the most important basic proteins are saliva rich pyrrolidinecarboxylic acid, glycoprotein PRB2, and an unknown protein with a level of 12.8; while the smallest proteins are T cell receptor 8 catenin fragment and hylaxin HNP-3, and the protein with a maximal relative molecular mass is mucoprotein 5B. Of the 309 saliva proteins identified according to

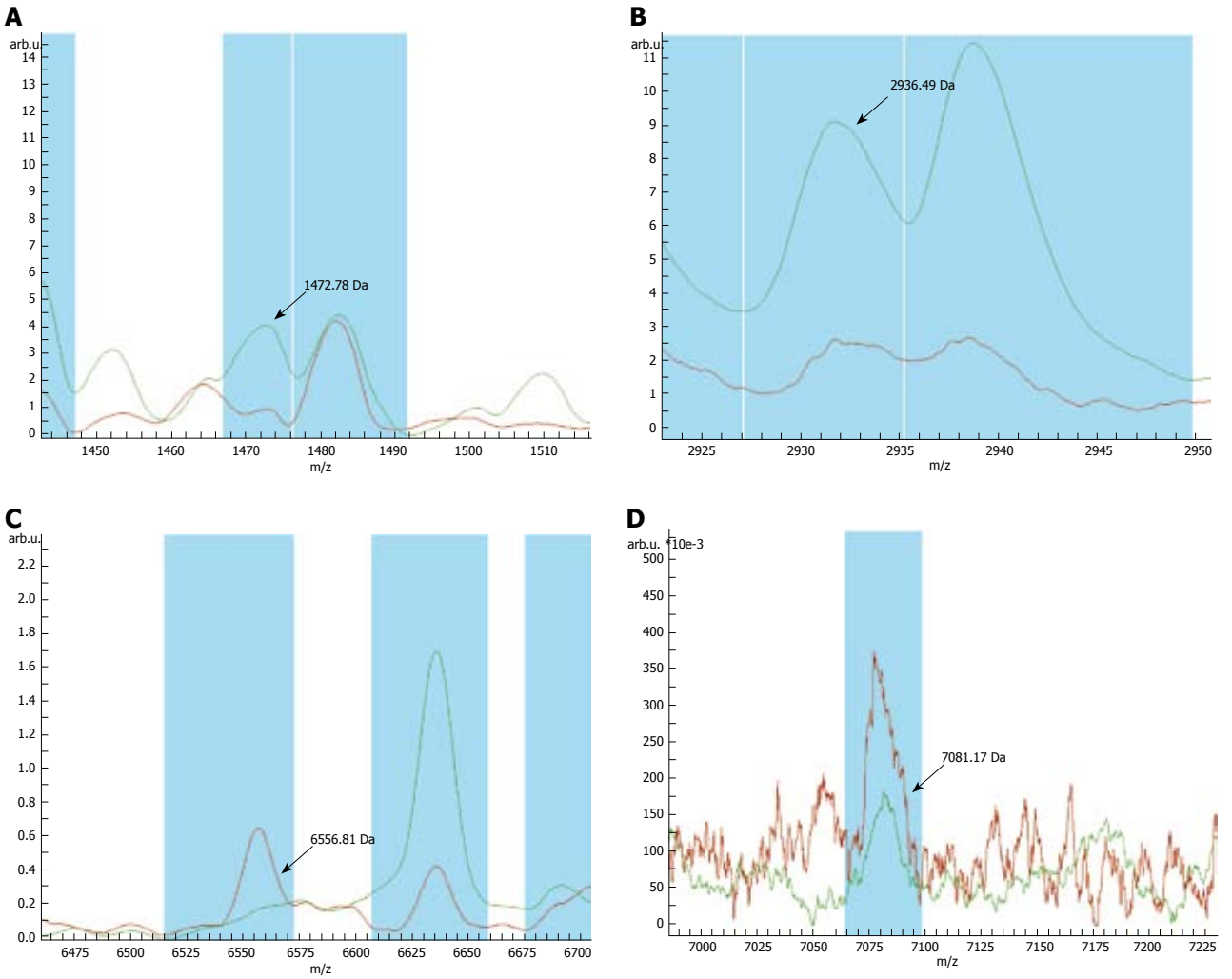

Figure 1 Sample spectrum showing a lower average peak value for proteins 1472.78 Da (A) and 2936.49 Da (B) in the normal group than in the gastric cancer group, and a higher value for proteins 6556.81 Da (C) and 7081.17 Da (D) in the normal group than in the gastric cancer group. Red line: Normal group; Green line: Gastric cancer group.

their functions, 28.7% are uncertain functional proteins, 21% are associated with immunity, 1.6% are associated with protein replication and reparation, 4.8% are associated with cell mobility and secretion, 2.3% are associated with transcription and ribosomes, 4.2% are associated cell multiplication and the cell cycle, 9.7% are associated with signal transduction, 5.2% are associated with metabolism, and 7.1% are associated with the cytoskeleton and endomembrane, respectively. Saliva is an important body fluid with complicated constituents and multiple biological functions. Its distinctive and abundant protein constituents undoubtedly can be used as biological markers of cancer and other diseases. The oral saliva protein flux analysis and precise determination have been restricted by old techniques, since the biological functions of most saliva proteins are unknown, and the value of saliva diagnosis and prognosis remains unclear. With the application of high-flux and high-precision proteomics, it has become possible that saliva proteins can be used as biological markers in prevention and bioprotein-targeted therapy, and in predicting the prognosis of diseases[3-10]. At present, no study on saliva proteomics is available in China, and synchronic detection and comparative study of saliva and serum proteins have not been reported worldwide.

Early stage markers of gastric cancer are a hot spot of investigation around the world. How to find effective and better methods and techniques that can be applied to the treatment of gastric cancer has become the focus of research. There are more than 1000 proteins in saliva, and saliva possesses atraumatic and convenient features, and can be used as a simple and abundant resource. In addition, recent studies have demonstrated that 20% of saliva proteins are similar in the blood, and certain saliva proteins are matched with blood proteins that influence senile dementia, breast cancer and diabetes[10-12]. MS technique is one of the most important techniques in proteomics studies. The most widely used MS technique in proteomics studies is MALDI-TOF MS. Application of MALDI-TOF MS, in study of biological markers of disease, is undoubtedly at the leading edge of molecular diagnosis. Proteomics has become a powerful tool for the discovery of new biological markers. Combining MS techniques and proteomics also provides good prospects for research of biological markers[13,14]. Investigation

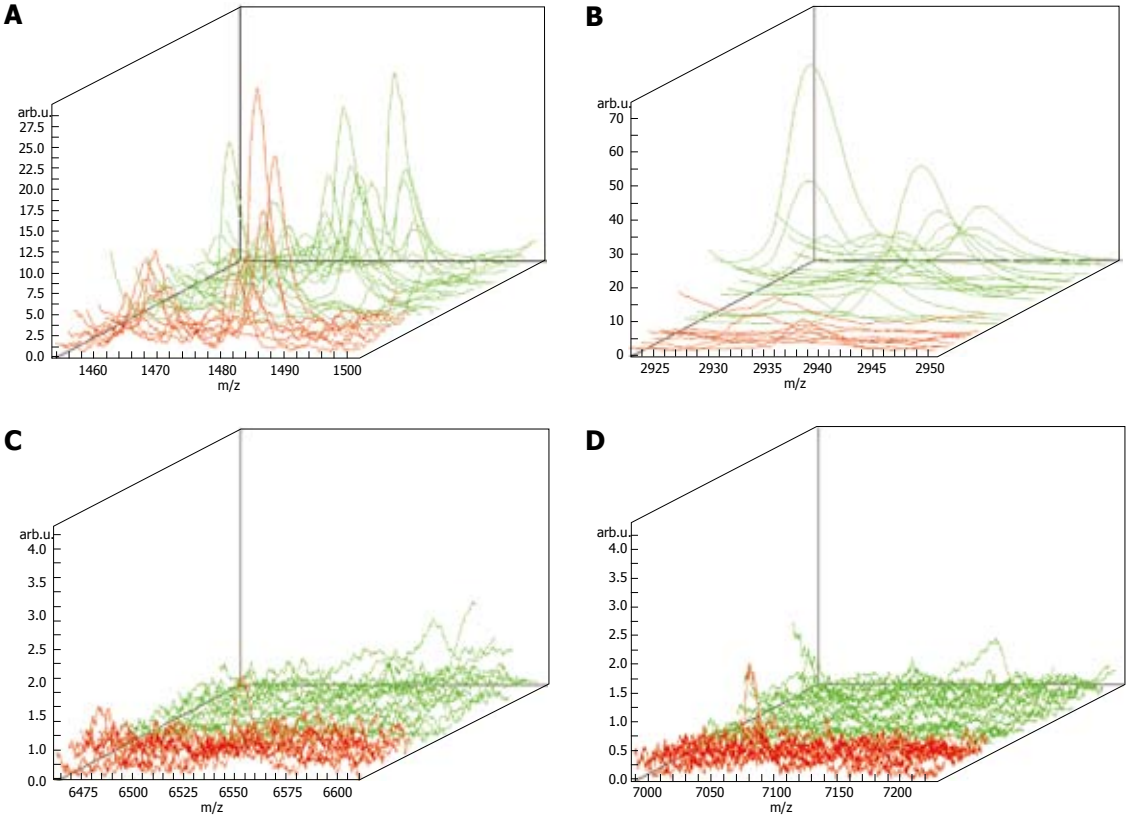

Figure 2 Three-dimensional map showing a lower average peak value for proteins 1472.78 Da (A) and 2936.49 Da (B) in normal group than in gastric cancer group, and a higher value for proteins 6556.81 Da (C) and 7081.17 Da (D) in normal group in gastric cancer group. Red line: Normal group; Green line: Gastric cancer group.

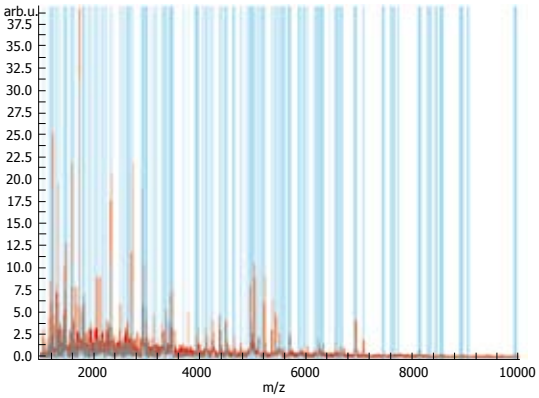

Figure 3 Complete mass spectrum of proteins 1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da, respectively.

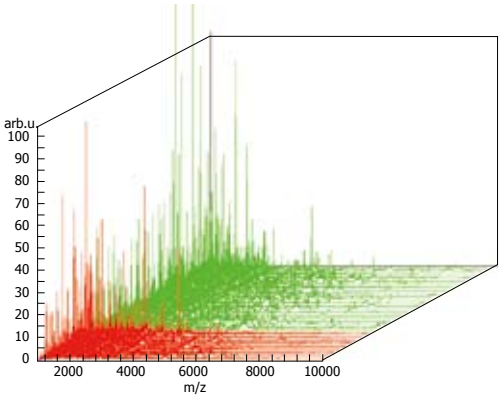

Figure 4 Complete three-dimensional mass spectrum of proteins 1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da, respectively. Red line: Normal group; Green line: Gastric cancer group.

of proteomics provides not only a material basis for vital movement rules, but also a theoretical basis for overcoming diseases. Certain diseases are related to special protein molecules. Proteins may be the molecular targets of new drugs or molecular markers for the early diagnosis of certain diseases. MS techniques, are important in studies of proteomics, and can be used in the study of protein/peptide spectra, biological marker spectra, or single biological markers for complicated diseases, such as cardiovascular and cerebrovascular disease, tumors, stroke and neuro-degenerative disease. Furthermore, they are also expected to open new avenues for research into pathogenesis, diagnosis and treatment of complicated diseases[15-17].

Gastric cancer usually has a long latent period. Early gastric cancer does not present with overt symptoms. Gastric cancer is at an advanced stage when it is diagnosed and has characteristics such as easy metastasis and poor prognosis. Therefore, early diagnosis is extremely important for the control and treatment of gastric cancer. Even though advanced diagnostic methods, such as X-ray barium meal examination, dual-phase helical computed tomography (CT), virtual CT, and gastroscopy, can improve gastric cancer diagnosis, their sensitivity and specificity are low for early discovery

of gastric cancer. Under such circumstances, gastric cancer can be found only after cancer cells have metastasized to their surrounding tissues, or whole-body aggravation occurs. Furthermore, cancer biological markers have a rather low positive rate for the early diagnosis of gastric cancer. At present, the sensitivity of cancer biological markers is only about 18%-40%. Combined examination of multiple cancer biological markers can achieve a sensitivity of 60%-80%. However, since the false positive rate is rather high, it is difficult to employ it as a biological index for the early diagnosis of gastric cancer[18-21]. We used the MALDI technique and WCX magnetic beads to examine gastric cancer samples, which can accurately identify gastric cancer. A difference in mass spectra was found between the normal subjects and gastric cancer patients, indicating that the present method can greatly improve the diagnosis of gastric cancer and might be used in its treatment. Twenty-three saliva samples from gastric cancer patients and 18 saliva samples from healthy volunteers were examined using MALDI-TOF-MS (AutoFlex Ⅲ, from German Bruker Company) and WCX magnetic beads. The mass peaks of four proteins (1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da) were significantly different in gastric cancer and normal groups. The mass peak of protein 1472.78 Da was significantly higher in the gastric cancer group than in the normal group, indicating that the protein of 1472.78 Da may play an important role in the occurrence and development of gastric cancer. Among the four peaks, only the mass peak of protein 6556.81 Da in samples from gastric cancer patients was lower than that in samples from normal subjects, suggesting that the protein expression mass spectrum diagnosis model for classification of gastric cancer and non-gastric cancer can be developed by comparative analysis of mass peaks of proteins 1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da, thus providing a new tool for predicting early gastric cancer. The positive identification rate for the mass peaks of these four proteins was significantly higher than that for the common serum cancer biological markers. Since the number of gastric cancer patients and normal controls was small, a larger sample size is needed to verify the mass peaks of the four proteins identified in this study.

In conclusion, the saliva proteome technique is an attractive prospect in the early clinical diagnosis of gastric cancer. Gastric cancer can be identified at an early stage by screening the high-risk group using MALDI technique and further studies on these proteins are needed to reveal their clinical value. Earlier detection and treatment of gastric cancer are important in decreasing the death rate. However, the saliva proteome technique needs to be further studied, because its cost is rather high and some other problems remain to be solved.

COMMENTS

Background

Gastric cancer is one of the most common cancers and threatens human health. At present, available tumor markers have a low sensitivity and a relatively high false-positive rate for the diagnosis of gastric cancer, thus cannot predict early gastric cancer. Therefore, it is necessary to find new methods to predict early stage gastric cancer.

Research frontiers

Saliva is an important and necessary body fluid. Studies on saliva proteomics have received extensive attention worldwide. At present, markers of early stage gastric cancer are a hot topic of investigation around the world. How to find effective and better methods that can be applied in clinical practice has become the target of many studies. Recent studies reveal that there are more than 1000 proteins in saliva, and saliva possesses atraumatic and convenient features, and can be used as a simple and abundant resource.

Innovations and breakthroughs

A diagnosis model was developed using weak cation exchange (WCX) magnetic beads to test saliva specimens from gastric cancer patients and healthy subjects. Significant differences in the mass to charge ratio peaks of four proteins (1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da) were observed between gastric cancer patients and healthy subjects.

Applications

The saliva proteomic model for distinguishing gastric and non-gastric cancer can be applied to the early diagnosis of gastric cancer.

Terminology

Saliva: An important and necessary body fluid. Blood constituents such as hormones, amino acids, electrolytes, immunoglobulin and creatinine *etc*, can enter saliva through the blood barriers of the capillary walls.

Peer review

The authors studied the differences in saliva protein spectra between normal subjects and gastric cancer patients. The study is valuable in aiding early diagnosis of gastric cancer. The method for screening patients with the risk of gastric cancer is simple, fast, and easy to use. Therefore, this study is innovative and practical, and the method developed by the authors can be used in the early diagnosis of gastric cancer.

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Combined treatment of oxaliplatin and capecitabine in patients with metastatic esophageal squamous cell cancer

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Abstract

AIM: To investigate the efficacy and side effects of the combined therapy of oxaliplatin and capecitabine in patients with metastatic esophageal squamous cell cancer (ESCC) and the survival of the patients.

METHODS: Sixty-four patients (median age of 63 years) with histological or cytological confirmation of ESCC received oxaliplatin 120 mg/m^2 intravenously on day 1 and capecitabine 1000 mg/m^2 orally twice daily on days 1 to 14 in a 21-d treatment cycle as palliative chemotherapy. Each patient received at least two cycles of treatment. The efficacy, side effects and patient survival were evaluated.

RESULTS: The partial response (PR) rate was 43.8% (28/64). Stable disease (SD) rate was 47.9% (26/64), and disease progression rate was 15.6% (10/64). The clinical benefit rate (PR + SD) was 84.4%. The main toxicities were leukopenia (50.0%), nausea and vomiting (51.6%), diarrhea (50.0%), stomatitis (39.1%), polyneuropathy (37.5%) and hand-foot syndrome (37.5%). No grade 4 event in the entire cohort was found. The median progression-free survival was 4 mo, median overall survival was 10 mo (95% CI: 8.3-11.7 mo), and the 1- and 2-year survival rates were 38.1% and 8.2%, respectively. High Karnofsky index, single metastatic lesion and response to the regimen indicated respectively good prognosis.

CONCLUSION: Oxaliplatin plus capecitabine regimen is effective and tolerable in metastatic ESCC patients. The regimen has improved the survival moderately and merits further studies.

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Key words: Oxaliplatin; Capecitabine; Metastatic esophageal squamous cell cancer; Survival analysis

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INTRODUCTION

Esophageal cancer, which has the highest incidence and mortality worldwide[1-4] is one of the most common malignant tumors in China. Linzhou (formerly known as Linxian) and nearby cities, such as Anyang and Huixian in Henan Province of northern China have been well recognized as the highest incidence area for esophageal squamous cell carcinoma (ESCC) in the world; the average incidence rates for men and women are 161 and 103 per 100 000, respectively[5].

Due to the lack of obvious early symptoms, the patients were often diagnosed at advanced stages, more than half of them with metastasis[6]. The recurrence and metastasis rate after treatment of esophageal cancer have the trend to ascend in recent years. In 2007, Grunberger *et al*[7] have confirmed that palliative chemotherapy can prolong the survival of stage Ⅳ

esophageal cancer patients, relieve their symptoms and improve their quality of life. Nevertheless, no optimizing chemotherapy regimen has been developed so far, the combined regimens based on cisplatin and 5-FU has been used frequently, with an effective rate of about 25.0%-33.0%[8,9]. Squamous cell esophageal cancer is the most common histology in China, and the constituent ratio is different from that in Europe and America. Some experts state that there are complete differences between esophageal adenocarcinomas and squamous cell cancer, such as the treatment protocol and prognosis. Therefore, the focus must be laid on the study of palliative chemotherapy of metastatic ESCC.

Oxaliplatin is a kind of chemotherapeutic drug belonging to the third generation of platinum compounds, which has played an important role in the treatment of colon cancer and other solid tumors[10,11]. Oxaliplatin's side chain is substituted by the diamino-cyclohexane radical (DACH). Therefore, compared to cisplatin, DACH-platinum combines to DNA much faster with stronger cell toxicity, which has no cross tolerance with cisplatin and no oto-renal toxicity. Furthermore, it has a synergistic effect with 5-FU, with slight digestive tract reaction and hematotoxicity. Its common side effect is reversible peripheral nerve paresthesia. Oral capecitabine can be rapidly absorbed as an intact molecule in the gastrointestinal tract and most of a given dose of capecitabine is initially hydrolyzed in the liver by a carboxylesterase to 5'-deoxy-5-fluorocytidine (5'-DFCR) without bioactivity. Cytidine deaminase, an enzyme found in many tissues, including tumors, converts 5'-DFCR to 5'-DFUR. Certain human carcinomas express the enzyme thymidine phosphorylase in higher concentrations than the surrounding normal tissues, which potentially converts 5'-DFUR to higher concentrations of active 5-fluorouracil (5-FU) within these tumors.

This study aims to explore the efficacy and toxic reaction of the combined treatment of oxaliplatin and capecitabine in metastatic ESCC and the survival of the patients. The results will be used to supply information and instruction for clinical treatment.

MATERIALS AND METHODS

Patients

From January 2003 to January 2006, 64 patients (45 males and 19 females) with histological or cytological confirmation of metastatic ESCC received oxaliplatin plus capecitabine therapy. The median age of the patients was 63 years (27cases under 60 years and 37 cases over 60 years). The metastatic sites of ESCC patients were lymph node, bone, liver, lung, membrana pleuralisa, abdominal membrane, adrenal gland, skin and soft tissue. Among these patients, 42 had single-site metastasis and 22 had multi-site metastases. Karnofsky performance status (KPS) of the patients was between 60 and 100 (60-80 in 42 patients and 90-100 in 23 patients). Before the study, 28 patients had received no chemotherapy, and 36 had received previous chemotherapy, and oxaliplatin and capecitabine treatment was excluded.

All patients were required to take pathological examinations, upper gastrointestinal tract barium meal perspective, computed tomography (CT) for neck thorax and abdomen, magnetic resonance imaging or CT for skull, emission computerized tomography for bone, blood routine test, liver-renal function test, electrocardiography (ECG) and other routine tests.

Treatment

All patients received oxaliplatin and capecitabine as follows: oxaliplatin 120 mg/m^2, infused on day 1; capecitabine 1000 mg/m^2, taken orally twice a day on days 1-14. Before taking oxaliplatin, the patients received 5-hydroxy-tryptamine inhibitors to prevent vomiting. During the medication, the patients should keep their body warm, avoid cold drinks, and take vitamin B6 100 mg orally three times a day with capecitabine to prevent and decrease the occurrence of extremity syndrome. Blood routine and liver-renal function tests should also be performed, and abnormal tests should be managed to accomplish the chemotherapy. Patients with bone metastasis should receive the radiotherapy and diphosphonate simultaneously in the 21-d cycle treatment. Each patient received at least two cycles of chemotherapy.

Evaluation criteria

After completion of two cycles of chemotherapy, all patients received overall check-up. Tumor response was assessed using Response Evaluation Criteria in Solid Tumors, such as the change of the tumor size, quantity and the appearance of new lesions. Toxicity was evaluated according to the Common Toxicity Criteria for acute and subacute toxicity reactions, and confirmed again at 4 wk after treatment. Patients benefited from the treatment complete response (CR) and partial response (PR) were given one or two more cycles of chemotherapy based on their agreement and tolerance. If the disease was progressive, they should receive other chemotherapeutic protocols, and optimized supportive treatment should be administered if the patients agree and are tolerant.

Follow-up

After completion of chemotherapy, all patients were followed up every 3 mo in the first year and every 6 mo in the second year by outpatient service and telephone interview till patients' death.

Statistical analysis

Overall survival, progression-free survival, death or last follow-up results were evaluated by the Kaplan-Meier method. The life table method was used to evaluate the 1-year and 2-year survival rates. Single factor was compared by log-rank test, and multi-factor was analyzed by Cox regression proportional hazard model.

RESULTS

Short-term effects

All patients were evaluated for short-term effects and toxicity. There was no CR; 28 patients (43.8%) had

Table 1 Toxicities of oxaliplatin plus capecitabine in 64 cases of metastatic ESCC (*n* %)

Side effects	Grade				
	0	Ⅰ	Ⅱ	Ⅲ	Ⅳ
Nausea and vomiting	31 (48.4)	21 (32.8)	12 (18.8)	0 (0.0)	0 (0.0)
Diarrhea	32 (50.0)	20 (31.3)	12 (18.7)	0 (0.0)	0 (0.0)
Aspherinia	44 (68.7)	11 (17.2)	9 (14.1)	0 (0.0)	0 (0.0)
Leukopoenia	32 (50.0)	20 (31.3)	10 (15.6)	2 (3.1)	0 (0.0)
Thrombocytopenia	46 (71.9)	11 (17.2)	7 (10.9)	0 (0.0)	0 (0.0)
Nerve toxicity	40 (62.5)	14 (21.9)	9 (14.1)	1 (1.5)	0 (0.0)
Hand-foot syndrome	40 (62.5)	13 (20.3)	11 (17.2)	0 (0.0)	0 (0.0)
Alopecia	60 (93.8)	4 (6.2)	0 (0.0)	0 (0.0)	0 (0.0)
Mucositis of mouth	39 (60.9)	18 (28.1)	7 (10.9)	0 (0.0)	0 (0.0)
Abnormal liver function	56 (87.6)	7 (10.9)	1 (1.5)	0 (0.0)	0 (0.0)

Table 2 Prognostic single factor analysis of ESCC

Prognostic factor	Number	Survival rate (%)		MST (mo)	*P*
		1-yr	2-yr		
Sex					0.713
Male	42	38.1	7.1	10.0	
Female	119	26.3	10.5	8.5	
Age (Yr)					0.887
< 60	26	30.8	11.5	9.0	
⩾ 60	35	42.9	5.7	10.0	
KPS					< 0.001
60-80	40	22.5	0.0	8.0	
90-100	21	66.7	23.8	13.5	
Metastasis					< 0.001
Mono-site	40	52.5	12.5	12.5	
Poly-site	21	9.5	0.0	6.5	
Prechemotherapy					0.969
Yes	27	40.7	3.7	10.0	
No	34	35.3	11.8	9.0	
Therapeutic effect					
PR	27	63.0	18.5	13.0	
SD	24	25.0	0.0	9.0	
PD	10	0.0	0.0	6.0	

PR, 26 patients (40.6%) demonstrated stable disease (SD) and 10 (15.6%) patients presented with cancer progression. The effectiveness rate was 40.6% and the overall clinical benefit rate was 84.4%.

Toxic and side effects

The main side effects of chemotherapy were alimentary tract reaction such as nausea, vomiting and diarrhea, and different grade bone marrow suppression. The occurrence of nausea and vomiting was 51.6%, and that of diarrhea was 50.0%. All side effects were slight or moderate. The main bone marrow suppression was leukopenia. The incidence rates of grade Ⅰ, Ⅱ, Ⅲ and Ⅳ leucopenia were 31.3%, 15.6%, 3.1% and 0.0%, respectively. One female patient had the symptom of digit anesthesia and anodynia late in the second cycle of chemotherapy, but this relieved gradually without treatment. The others only had grade Ⅰ or Ⅱ nerve toxicity such as dead limb, dysesthesia, and cold sensitivity. All of these recovered soon during the intermission of chemotherapy. No severe hand-foot syndrome occurred. The incidence rate of slight and moderate hand-foot syndrome was 37.5%. The other toxicities were grade Ⅰ or Ⅱ tolerable oral mucositis (39.1%), liver function abnormality (1.4%) and alopecia (6.2%). There was no renal function abnormality and death related to chemotherapy (Table 1).

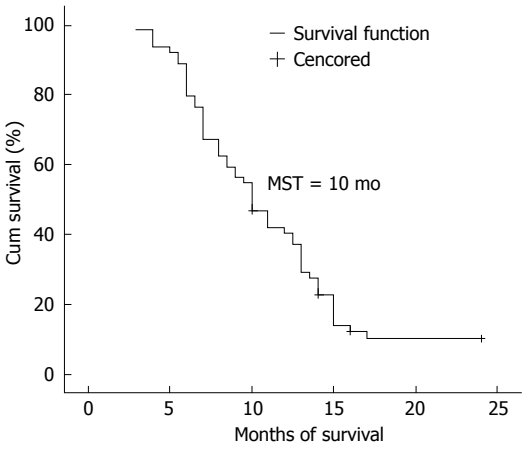

Figure 1 Cumulative survival curve of ESCC patients.

Survival analysis

The 64 patients were all followed up either for 2 years or until death. The follow-up rate was 100.0% (Figure 1). The median progression-free survival was 4.0 mo, and the median overall survival was 10.0 mo (95% CI: 8.3-11.7 mo). The 1- and 2-year survival rates were 38.1% (24/63) and 8.2% (5/61), respectively. Kaplan-Meier monofactorial analysis indicated that there was a statistical significance between the influence of KPS index, metastasis and short-term effect and survival ($P \leqslant 0.0001$), but there was no statistical significance between the influence of sex, age and therapy and survival ($P > 0.05$), (Figure 2). Cox regression proportional hazard model polyfactorial analysis indicated that KPS index, the number of tumor metastasis loci and short-term effect ($P < 0.001$) were independent survival prognostic factors, while sex, age and former therapy ($P > 0.05$) were not (Tables 2 and 3).

DISCUSSION

It is very important to treat enteric tumors by oxaliplatin plus capecitabine. There are few reports about this protocol used in esophageal cancer[12,13]. As a result of different pathological types, there have been some reports about combined treatment of oxalipatin and capecitabine for esophageal adenocarcinoma. However, there has been no report about this protocol for ESCC. Compared with other treatment of advanced ESCC, our effectiveness rate is slightly lower than that of protocol of paclitaxel and cisplatin reported by Huang *et al*[14], but higher than that of combined regimens based on cisplatin and 5-FU, as well as irinotecan and cisplatin, and similar to that of FOLFOX 4. The median overall survival is longer and the 1-year survival rate is a little higher in our study than the regimens based on cisplatin and 5-FU, as well as FOLFOX 4, both of which are frequently applied clinically. Moreover, our regimen has fewer side effects.

Table 3 Results of proportional hazards regression model

Factor	Regression coefficient	Standard error	Wald	DOF	*P*	Exp (β)	95% CI
Sex	-0.439	0.361	1.475	1	0.225	0.645	0.318-1.309
Age	-0.151	0.342	0.194	1	0.659	0.860	0.440-1.682
KPS	-1.449	0.342	17.906	1	< 0.001	0.235	0.120-0.459
Metastasis	1.932	0.390	24.497	1	< 0.001	6.902	3.212-14.833
Pre-chemotherapy	-0.235	0.291	0.653	1	0.419	0.790	0.447-1.398
Short-term effect	0.972	0.254	14.610	1	< 0.001	2.645	1.606-4.354

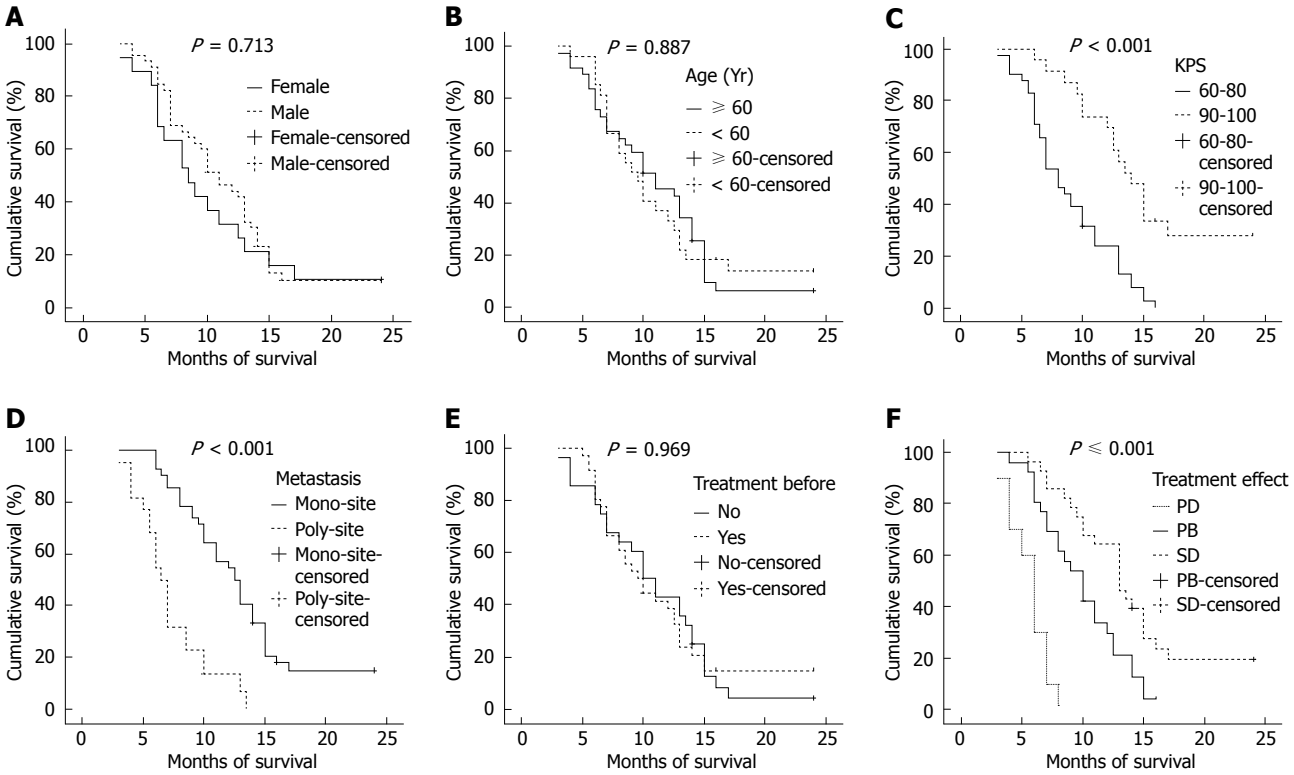

Figure 2 Cumulative survival curve. A: Cumulative survival curve of male and female ESCC patients; B: Cumulative survival curve of ESCC patients at different ages; C: Cumulative survival curve of ESCC patients with different KPS; D: Cumulative survival curve of ESCC patients with poly/mono-site metastasis; E: Cumulative survival curve of ESCC patients with or without pre-treatment; F: Cumulative survive curve of ESCC patients with different short-term effects.

Mauer *et al*[10] reported that oxaliplatin and 5-FU protocol (oxaliplatin 85 mg/m^2 iv, 5-FU 400 mg/m^2 iv quickly and then 600 mg/m^2, iv for 22 h, on day 1 and 2), has better results. The PR rate was 40.0%, the median overall survival was 7.1 mo, and 1-year survival rate was 31.0%. The main toxicities were neutrocytopenia (grade Ⅳ, 29.0%,) and peripheral neuropathy (grade Ⅱ-Ⅲ, 26.0%).

Huang *et al*[14] used paclitaxel and cisplatin regimen (paclitaxel 175 mg/m^2 , iv less than 3 h on day 1; cisplatin DDP 40 mg/m^2, iv on day 2 and 3; and repeated every 3 wk), with a PR rate of 55.5%. Of seven patients with severe neutrocytopenia, one patient died of grade Ⅳ neutrocytopenia.

Polee *et al*[15] used cisplatin, etoposide and 5-FU regimen (cisplatin DDP 80 mg/m^2, iv on day 1; etoposide 125 mg/m^2, iv on day 1 and 200 mg/m^2 , iv on day 3 and 5; 5-FU 375 mg/m^2, iv on days 1-4; folic acid 30 mg, taken orally every 4 h, on days 1-4; and the cycle was repeated every 4 wk), the PR rate was 34.0%, the median overall survival was 9.5 mo, and the 1-year survival rate was 36.0%. The main toxicities were leukopenia (grade Ⅲ-Ⅳ, 16.0%), fever related to leukopenia (19.0%), thrombocytopenia (grade Ⅲ-Ⅳ, 7.0%), mucositis (grade Ⅲ-Ⅳ, 23.0%), nausea and vomiting (grade Ⅲ, 32.0%) and diarrhea (grade Ⅲ, 6.0%).

Lorenzen *et al*[16] used capecitabine (1000 mg/m^2 taken orally twice daily on days 1-14) plus intravenous docetaxel (75 mg/m^2 on day 1). The median survival was 15.8 mo (95% CI, 7.8-23.9 mo). The intent-to-treat efficacy analysis showed an overall response rate (ORR) of 46.0%.

Lee *et al*[17] used 60 mg/m^2 of CDDP iv on day 1 and capecitabine 1250 mg/m^2 taken orally twice a day on days 1-14. The ORR was 57.8% (95% CI, 43.3-72.2). The median duration of response was 4.6 (1.0-15.6) mo, follow-up of 25.7 (10.8-42.6) mo, progression time of 4.7 mo (95% CI: 2.5-7.0 mo) and the median survival time was 11.2 mo (95% CI: 8.5-13.9 mo).

Lin *et al*[18] used the regimen, composed of paclitaxel 35 mg/m^2 1 h iv on day 1, 4, 8 and 11; cisplatin 20 mg/m^2, 2 h iv on day 2, 5, 9 and 12; and 5-FU 2000 mg/m^2, leucovorin 300 mg/m^2 24 h iv on day 5 and 12; and repeated every 21 d. The median progression-free and

overall survival rates were 6.3 and 8.9 mo, respectively.

Evans *et al*[19] used docetaxel and oxaliplatin on day 1 and 8 and capecitabine individually, twice daily, on day 1-10, with each cycle repeated every 21 d. The docetaxel dose ranged from 30 to 35 mg/m^2, the oxaliplatin from 40 to 50 mg/m^2, and the capecitabine from 750 to 850 mg/m^2 twice daily. Grade 3/4 dose-limiting toxicities of diarrhea, nausea, fatigue and febrile neutropenia occurred in three of four patients at dose level 3. An intermediate dose was added (2A) and the capecitabine dose reduced to 750 mg/m^2. One of 6 patients had a dose-limiting toxicity at level 2A.

Tsai *et al*[20] used carboplatin (area under the ROC curve AUC = 2) on day 1 and 8, docetaxel (35-40 mg/m^2) on day 1 and 8, and capecitabine (500-2000 mg/m^2) on days 1-10. The maximum tolerated dose of docetaxel was 40 mg/m^2 on day 1 and 8; carboplatin, AUC = 2 on day 1 and 8; and capecitabine, and 1500-2000 mg/m^2 on days 1-10 in a 21-day cycle. Ten of 25 patients who could be evaluated (40.0%) responded and eight of 14 patients treated at the final dose level responded (57.0%).

Lee *et al*[21] used two cycles of XP induction chemotherapy, consisting of capecitabine 1000 mg/m^2 twice daily on days 1-14, and cisplatin 60 mg/m^2 iv on day 1, every 3 wk. Patients classified as M1a and M1b (non-visceral lymph node metastases) were treated with 54 Gy radiotherapy, concurrently with weekly capecitabine 800 mg/m^2 twice daily on days 1-5 and cisplatin 30 mg/m^2 iv on day 1 during radiation. Patients classified as M1b (visceral metastases) were treated with chemotherapy only until disease progression or intolerance to chemotherapy. The median time of progression was 7.8 mo (95% CI, 6.0-9.5 mo) and the median overall survival was 12.0 mo (95% CI, 9.0-15.0 mo).

Evans *et al*[22] used a regimen comprised of docetaxel 40 mg/m^2, on day 1 and 8, carboplatin (AUC = 2) on day 1 and 8, and capecitabine 2000 mg/m^2, on days 1-10 in a 21-day cycle. The median survival was 8.0 mo (95% CI, 5.5-13.0 mo), and the 1-year survival rate was 36.0%.

In our study (oxaliplatin 120 mg/m^2, iv on day 1; capecitabine 1000 mg/m^2, taken orally twice a day on days 1-14; and repeated every 3 wk), the rate was 43.8%, the median overall survival was 10 mo, and the 1-year survival rate was 38.1%. The main toxicities were leukopenia (grade Ⅲ, 31.0%) and neuro-toxicity (grade Ⅲ, 1.5%).

Capecitabine can be taken orally, so the protocol has superiority in medication. The mono-factorial analysis by Kaplan-Meier indicates that patients with low KPS and multi-locus metastases benefit little from this therapy. The short-term effect indicates that the prognosis demonstrates the importance of prompt, objective and precise therapeutic effect in clinical practice. Cox regression proportional hazard model poly-factorial analysis indicates that KPS index, the number of tumor metastasis locus and short-term effect are independent survival prognostic factors.

Our results demonstrate that oxaliplatin plus capecitable regimen has the advantage of good short-term effects, convenient administration and minor side effects in metastatic ESCC. The functional status of prior treatment, the number of tumor metastasis loci and short-term effects are independent survival prognostic factors.

COMMENTS

Background

Esophageal cancer which has the highest incidence and mortality worldwide is one of the most common malignant tumors in China. Esophageal squamous cell cancer (ESCC) is the most common histology. It has been confirmed that palliative chemotherapy can prolong the survival of stage Ⅳ esophageal cancer patients, relieve their symptoms and improve their quality of life. Nevertheless, no optimizing chemotherapy regimen has been available so far, the combined regimens based on cisplatin and 5-fluorouracil have been used frequently, but the effectiveness rate is only about 25.0%-33.0%.

Research frontiers

Oxaliplatin is a kind of chemotherapeutic drug belonging to the third generation of platinum compounds, which has played an important role in the treatment of colon and rectum cancer and other solid tumors. It has a synergistic effect with lesser digestive tract reaction and hematotoxicity. Capecitabine, which has milder side effects, can be taken orally and is rapidly absorbed as an intact molecule in the gastrointestinal tract. Therefore, the combined regimen of oxaliplatin and capecitabine may produce more clinical benefits.

Innovations and breakthroughs

This study explored the efficacy and toxic reaction of oxaliplatin plus capecitabine in the treatment of patients with metastatic ESCC and the survival of the patients. The partial response (PR) rate was 43.8% (28/ 64). Stable disease (SD) rate was 47.9% (26/64), and disease progression rate was 15.6% (10/64). The clinical benefit rate (PR + SD) was 84.4%. No grade Ⅳ side effect in the entire cohort was found. The results can be used to supply information and instruction for clinical treatment.

Applications

Higher response and survival rate, and lower rate of toxicity were obtained by the combined treatment in this study. Capecitabine can be taken orally, therefore, that this treatment can be used clinically.

Terminology

RECIST stands for Response Evaluation Criteria in Solid Tumors. KPS stands for Karnofsky performance score.

Peer review

This is the first report to examine the efficacy and toxicity of the combined therapy of oxaliplatin and capecitabin in patients with metastatic ESCC. Higher response and survival rate, and lower rate of toxicity were obtained by this treatment than the other treatment protocols reported previously. It seems that this treatment has become a candidate for phase Ⅲ study. Furthermore, since this treatment can be given on an outpatient basis, this study has great value.

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CASE REPORT

An obstructing mass in a young ulcerative colitis patient

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Abstract

We present a case of a 19-year-old female who developed subacute obstruction due to giant inflammatory polyps, having undergone treatment for left-sided ulcerative colitis. This is followed by a review of the literature.

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Key words: Pseudopolyp; Ulcerative colitis; Inflammatory polyps; Giant intestinal polyposis

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INTRODUCTION

Giant inflammatory polyps occur rarely in ulcerative colitis patients. The presentation is often insidious and the endoscopic appearance can be alarming. The following case illustrates these points.

CASE REPORT

A 19-year-old female student presented to Hemel Hempsted General Hospital in May 2006 following a 20-d history of bloody diarrhea. The patient was on no regular medications at the time of admission. She had no significant past medical history apart from iron deficiency anemia [hemoglobin 7.4 g/dL, mean corpuscular volume (MCV) 67.3] with raised inflammatory markers (C-reactive protein; CRP, 89 mg/L). Flexible sigmoidoscopy demonstrated left-sided colitis and subsequent biopsies confirmed it to be ulcerative colitis. She received a short course of oral prednisolone (40 mg) and balsalazide (2.25 mg *tds*) with rapid improvement. At the time of her follow-up appointment in July 2006, she was asymptomatic.

At a subsequent clinical appointment in September 2006, the patient complained of increasing nausea, lethargy and borborygmi. Blood taken at the time revealed a microcytic anemia (hemoglobin 9.8 g/dL, MCV 78) despite being on ferrous sulfate and an albumin of 19 g/L. Her CRP was < 3 mg/L. She was readmitted to our hospital in November 2006 having developed 10 episodes of watery diarrhea a day. She was treated with steroids for exacerbation of colitis, started on azathioprine and discharged from our hospital. Over the subsequent months, the patient continued to complain of worsening lower abdominal pain, vomiting and lethargy. She was readmitted for investigation and her admission blood analysis revealed CRP < 3 mg/L. Her abdominal X-ray was unremarkable. She underwent a flexible sigmoidoscopy to assess the extent of inflammation. There was no evidence of active ulcerative colitis, but the discovery of a mass at the splenic flexure prompted further imaging with computed tomography (Figure 1). Because of the obstructive nature of the mass and the fact that the mass extended along the transverse colon, the patient underwent an extended right hemicolectomy with primary anastomosis (Figure 2). The histology of the mass revealed it to be an inflammatory pseudo-polyp with no evidence of dysplasia. She subsequently made a good postoperative recovery with a normalization of all blood results.

DISCUSSION

Giant inflammatory polyps (GIPs), also known as filiform polyposis or pseudo-polyps, are defined as being more than 1.5 cm in diameter. First described in 1965[1,2], they can occur in both ulcerative colitis and Crohn's disease,

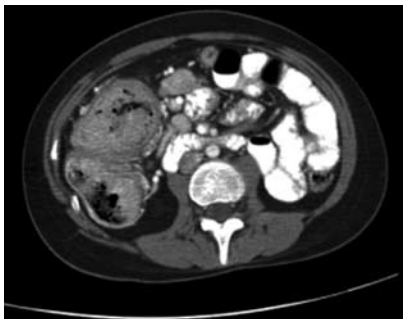

Figure 1 Abdominal computed tomography showing a mass at the splenic flexure.

Figure 2 Resected specimen revealing an inflammatory pseudo-polyp with no evidence of dysplasia.

although the former is more common. They occur most commonly in females with pancolitis at the age of 20-40 years, diagnosed 1-5 years prior to presentation with GIPs. There is a predilection for the transverse colon, although the condition has been described at all colonic sites. GIPs can present in a number of different ways, including crampy abdominal pain, anemia, obstruction, hypoproteinemia and palpable abdominal mass[3-8].

Its presentation and endoscopic findings may mimic those of a colonic tumor. There are no pathognomonic signs to confidently differentiate colonic pseudo-polyp clinically, radiologically or endoscopically from villous adenoma, dysplasia-associated lesion or mass or carcinoma[9]. The pathogenesis is deemed to be abnormal healing in the form of enthusiastic post-inflammatory regeneration[9-11]. GIPs have been found in both quiescent and active diseases[12] which may represent detection at different stages in their development. Balazs has found further evidence for this[13] from the histopathological analysis of GIPs which shows changes similar to those described in delayed type hypersensitivity[14]. Treatment is currently surgical in all previously described case reports, as most of the patients present with obstruction, and because of the size of the polyp.

We believe that GIPs should be suspected more often in young patients with colitis presenting with obstruction. Hypoalbuminemia is an interesting aspect that has been previously reported and attributed to an etiology similar to that of Menetrier's disease. Although non-specific in the appropriate clinical context, its presence should add further suspicion for the presence of GIPs[15,16].

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CASE REPORT

Extensive hepatic-portal and mesenteric venous gas due to sigmoid diverticulitis

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Abstract

Hepatic portal venous gas is most often associated with extensive bowel necrosis due to mesenteric infarction. Mortality exceeds 75% with this condition. The most common precipitating factors include ischemia, intra-abdominal abscesses and inflammatory bowel disease. In this report, we present a 75-year-old woman with extensive hepatic portal and mesenteric venous gas due to colonic diverticulitis. She had a 10-year history of type Ⅱ diabetes mellitus and hypertension. She was treated by sigmoid resection and Hartmann's procedure and discharged from the hospital without any complications.

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Key words: Hepatic portal vein; Gas; Sigmoid diverticulitis; Computed tomography

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INTRODUCTION

Hepatic portal venous gas (HPVG) is a rare condition and traditionally regarded to be an ominous finding of impending death, with highest mortality reported in patients with underlying bowel with ischemia[1-6]. Colonic diverticulitis is an inflammatory condition and in very rare cases can be associated HPVG[6-9]. HPVG can be due to two mechanisms: gas under pressure in the bowel lumen or to an alteration of the mocosa, allowing the gas to enter the portal system through the mesenteric veins; or gas-forming bacteria in intra-abdominal abscesses with or without a related pylephlebitis[3-6]. If there is an underlying intramesocolic abscess and perforation in complicated diverticulitis, the prognosis is favorable, but the prognosis of HPVG due to septic thrombophlebitis and gas-forming organisms is poor[6]. Another factor affecting HPVG and its prognosis is the existence of a long-term chronic disease, such as chronic renal failure, diabetes mellitus and hypertension[5]. It has been reported that long-term chronic diseases decrease immune functions and alter the intestinal microbial flora and tolerance capability of the HPVG patients, which might lead to fatality[5,10].

We report the case of a 75-year-old woman with type Ⅱ diabetes mellitus and hypertension presenting with extensive hepatic, portal and mesenteric venous gas due to sigmoid diverticulitis.

CASE REPORT

A 75-year-old woman was seen in the emergency department with a 4-d history of mild abdominal pain and fever. Except for her temperature (38.2℃), her vital signs were normal. She had a 10-year history of hypertension and type Ⅱ diabetes mellitus, and antihypertensive drugs and insulin therapy had kept her blood pressure and blood sugar level within normal ranges. On physical examination, she had mild tenderness in the left lower quadrant but no localizing peritoneal signs. Her serum C-reactive protein level was 220 mg/L, her platelet count was 55 000/mm^3, and other laboratory findings were normal. A computed tomography (CT) scan of the abdomen revealed multiple gas foci in the main hepatic-portal vein, portal vein branches (Figure 1) and superior mesenteric (Figure 2A), splenic (Figure 2B) and inferior

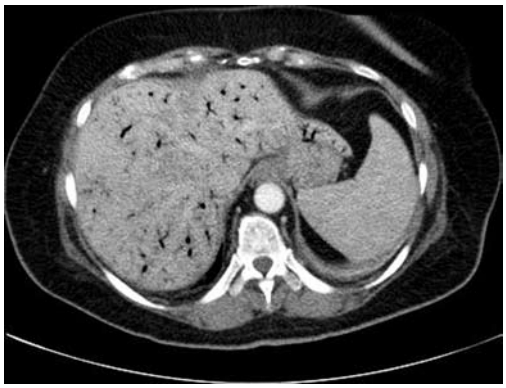

Figure 1 Extensive gas accumulation in the hepatic-portal vein branches.

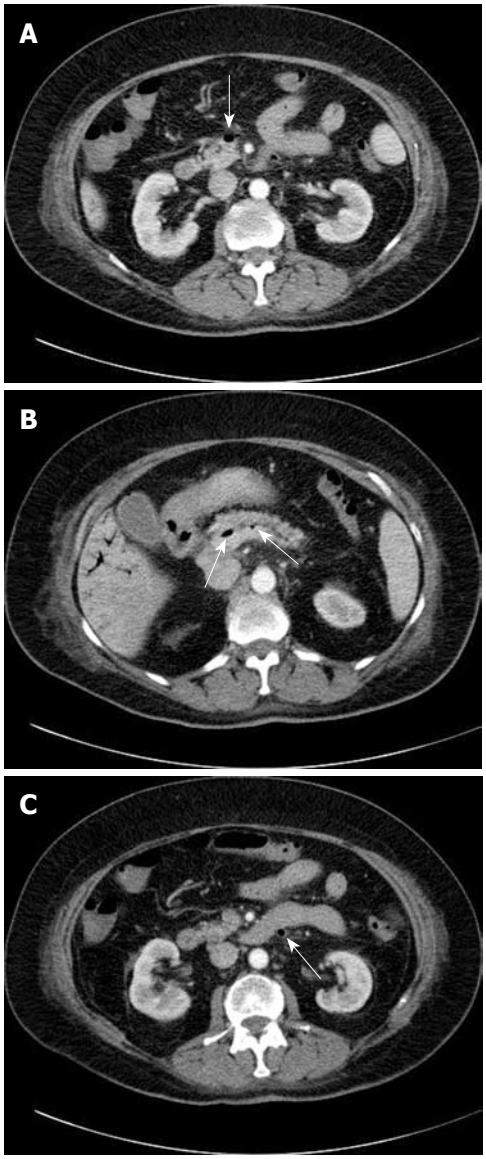

Figure 2 Gas observed. A: Superior mesenteric vein (arrow); B: Splenic vein (arrows); C: Inferior mesenteric vein (arrow).

mesenteric veins (Figure 2C). Sigmoid diverticulosis involved the mid and distal portions of the sigmoid colon. Slight wall thickening and inflammation suggesting localized sigmoid diverticulitis were observed (Figure 3).

Figure 3 Diverticulosis in the sigmoid colon and mild inflammatory changes are present in the sigmoid mesocolon (arrows).

The patient was transferred to the general surgery department, started on intravenous fluid therapy and antibiotics (ceftriaxone 2 g/d and ornidazol 1 g/d), and a laparotomy was planned for the same day. The emergency laparotomy revealed mild findings of diverticulitis in the sigmoid colon. A Hartmann's procedure and end colostomy were performed. The patient was discharged on the seventh postoperative day without further complications. Her colostomy was closed 8 wk after the first operation, and she reported no problems upon examination after 3 mo.

DISCUSSION

HPVG gas was first described by Wolfe and Evans[11] in 1955 in fatal necrotizing enterocolitis in infants. This entity is most commonly associated with ischemic bowel disease. In the series of 64 patients reviewed by Liebman *et al*[1], HPVG was mainly associated with necrotizing bowel disease (72%) and found to have a 75% mortality rate. HPVG venous gas has also been associated with such entities as bowel distention, perforated ulcer, acute hemorrhagic pancreatitis, corrosive ingestion and inflammatory bowel disease such as Crohn's disease[6,12-15].

HPVG and thrombophlebitis is a rare complication of diverticulitis. Zielke *et al*[3] reported that this entity was confined to nine patients with mesocolic abscesses. In 2007, Sellner *et al*[6] reported a second review of 21 patients with complicated diverticulitis because of HPVG. In this report, 55% of cases had mesocolic abscesses and in the remaining 45%, mesocolic abscesses were absent and the patients presented with septic pylephlebitis. The authors suggested that patients with mesocolic abscess have better prognosis than patients with septic pylephlebitis. Despite our patient's symptoms, the clinical findings of diverticulitis were very slight. The CT findings revealed extensive multiple gas foci in the main portal vein, portal vein branches and superior mesenteric vein, and also revealed that the diverticulitis was milder than expected. We thought that this discordance might be due to virulence of pathogens or deficiency of the patient's immune system as a consequence of diabetes mellitus. Therefore, we decided

to perform a laparotomy on the same day. Operational findings of diverticulitis were quite limited and slight and we did not observe a mesocolic abscess. Nobili *et al*[7] suggested that if medical conservative therapy could be resolutive and the clinical status improves, the surgery could be delayed in these patients. Even though the clinical status of our patient was stable, we preferred to perform an urgent laparotomy because we thought that prognosis might be worse due to diabetes mellitus and gas-forming organisms. Chan *et al*[5] have reported that long-term chronic diseases change the microbial flora in the intestine and decrease the immune function and tolerance capability of HPVG patients, which might lead to fatality. Our patient had type Ⅱ diabetes mellitus and hypertension for 10 years, and extensive HPVG might be explained by increased aerobic and anaerobic microorganisms in the intestinal flora due to diabetes mellitus.

In conclusion, the clinical and radiological findings of HPVG associated with diverticulitis may be variable. We thought that, although the clinical status of our patient was stable, extensive HPVG in diverticulitis could be dangerous, due to a compromised immune system and alteration in intestinal flora, especially among elderly patients with chronic systemic diseases and no obvious mesocolic abscess or perforation, such as in our patient.

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CASE REPORT

A rare case of duodenal duplication treated surgically

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Abstract

Duodenal duplication, a rare congenital malformation, can also be observed in adulthood. Although it can be cystic or tubular, communicating or non-communicating, cystic and non-communicating forms are the most common. Several complications, such as obstruction, bleeding, perforation and pancreatitis, may result. Optimal treatment is total excision, although endoscopic procedures have also been described in appropriate cases. If total excision is not possible, subtotal excision and internal derivation can be performed. The 38-year-old woman presented here had occasional attacks of abdominal pain and obstruction, and we considered the diagnosis of duodenal duplication by abdominal computerized tomography. As we confirmed the diagnosis with operative findings and histopathological signs, we treated her with subtotal excision and intraduodenal cystoduodenostomy.

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Key words: Duodenum; Duplication; Subtotal excision; Intraduodenal cystoduodenostomy

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INTRODUCTION

Duplications of the gastrointestinal system are rare congenital malformations observed in one out of 25 000 deliveries. About 33% of the cases are reported in adults above 20 years of age. Duodenal duplication constitutes 5%-7% of all gastrointestinal duplications. Its etiology is as yet unknown. Treatment is mainly surgical and total excision, if possible, is the procedure of choice. However, in some cases, alternative procedures, such as subtotal removal or digestive derivation, are required because of extensive size or location[1]. Here, we present a rare case of duodenal duplication in which the treatment was subtotal excision with intraduodenal cystoduodenostomy.

CASE REPORT

A 38-year-old woman who had occasional abdominal pain was referred to us with a clinical diagnosis of gastric outlet obstruction. The epigastrium was mildly sensitive on physical examination. Laboratory findings were normal but abdominal ultrasonography (US) showed gastric distension. Initial endoscopy was useless because of remnants of food. After nasogastric decompression, we repeated endoscopy but found no abnormalities. Upon abdominal computerized tomography (CT), a cystic lesion of 5 cm × 8 cm × 9 cm in diameter was observed, which extended along the lateral wall of the first and second parts of the duodenum. Remnants of food and orally taken contrast media were found within the lesion, and we observed the nasogastric tube entering the lesion through a defect between the duodenum and the cyst (Figure 1). We operated on the patient and found a cystic dilatation, 10 cm × 12 cm in diameter, anterolateral to the first and second parts of the duodenum (Figure 2A). We performed cystotomy and observed it make contact with the normally located duodenum at the posteromedial side of the cyst, through a defect of 2 cm × 2 cm in diameter. The wall between the duodenum and the cyst beneath the opening was covered with mucosa on both sides (Figure 2B). Thus, the diagnosis was cystic and communicating duodenal duplication. The wall between

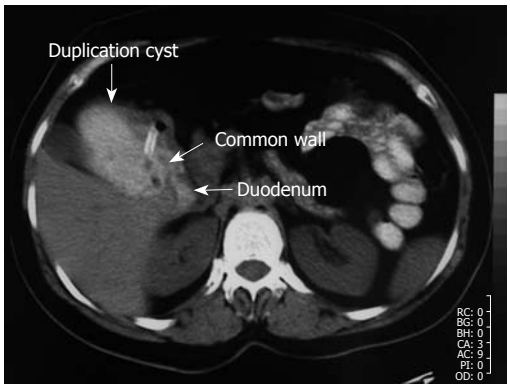

Figure 1 Oral and intravenous contrast-enhanced abdominal CT imaging. The nasogastric tube was extended into the cyst, which was filled with oral contrast medium, through the defect between the duodenum and the cyst.

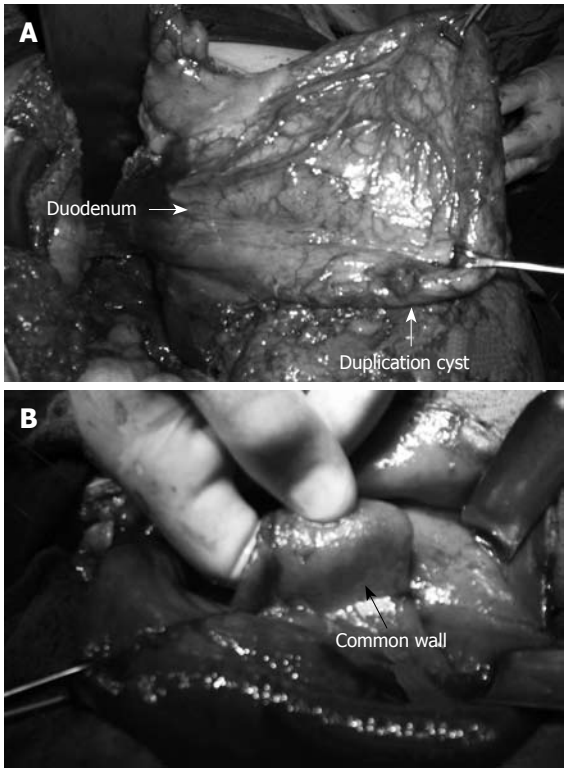

Figure 2 Operative view of cyst. A: Duodenal duplication cyst; B: Inner surface of the cyst.

the cyst and duodenum was excised and the corners were sutured to form a cystoduodenostomy. The cystic wall is excised next to the pylorus above, duodenum laterally, pancreas medially and the third part of duodenum below, while the remnant is sutured primarily. The diagnosis was confirmed histopathologically by identifying a separate mucosa with its own muscularis mucosa on both sides of the wall between the cyst and duodenum and intervening connective tissue fibers (Figure 3). The patient was without complaint after 9 mo follow-up.

DISCUSSION

Duplications of the gastrointestinal system can be observed anywhere along the alimentary tract, and they

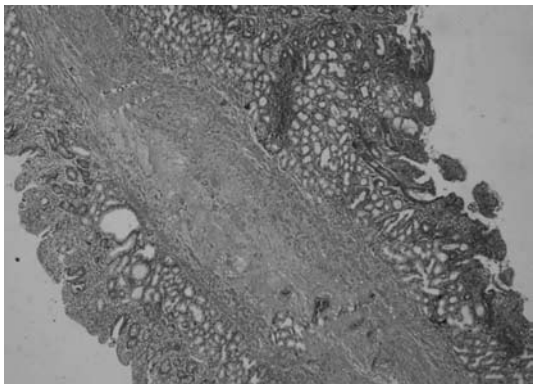

Figure 3 Common wall containing double mucosa with muscularis mucosa on each side and intervening connective tissue fibers (Hematoxylin and eosin, x 10).

are located most often in the ileum and least often in the duodenum. Duodenal duplications can be cystic or tubular, communicating or non-communicating, but the most common type is cystic and non-communicating. These are generally located at the medial border of the first and second parts of the duodenum and extend to the anterior or posterior side[1-3]. Duodenal duplication observed in our case was cystic and located in the first and second parts of the duodenum, but it was of the communicating type and located on the antimesenteric side.

A variety of clinical manifestations have been reported that are determined by the type, site and size of the duplication. Generally, patients present with a palpable mass in the abdomen, signs of intestinal obstruction, or abdominal pain. Bleeding or perforation caused by peptic ulcer and jaundice, and pancreatitis caused by biliary obstruction may also be the manifestations[1-6]. In the current case, occasional attacks of abdominal pain and gastrointestinal obstruction were present. Obstruction in non-communicating cystic duplications is defined by compression of the duplication cyst, which is distended by intracystic secretions[1]. However, in our case, which was of the communicating type, it can be described by compression of the cyst, which was filled with the gastric contents but not drained.

Although radiological methods are helpful for diagnosis, preoperative diagnosis of duodenal duplication is rarely made accurately. In barium studies, in non-communicating cysts, the first and the second parts of the duodenum can be seen as compressed and displaced by a mass, whereas, in the communicating type, the cyst itself can be observed as being filled with barium[2,7]. In the current case, if barium studies had been performed, the communication between the duplication and duodenum would have been demonstrated better. Duodenal duplication is differentiated from other cystic lesions by the "gut signature" of its wall observed by abdominal or endoscopic US. Gut signature refers to the layered pattern of the wall, with the hyperechoic inner layer representing the submucosa and the hypoechoic outer layer representing the smooth muscle[8-10]. Peristalsis

of the cyst wall noted upon real-time US is strongly suggestive of a duplication cyst[11]. US is an operator-dependent method and unfortunately it was not helpful in the diagnosis of our case. CT is valuable in identifying the type, location and the size of the duplication cyst. In the differential diagnosis of duodenal duplication, one should be mindful of choledochocele, pancreatic pseudocyst and intraluminal diverticulum[12]. In our case, the location of the cystic lesion and CT images made us think of cystic, communicating duodenal duplication. Although magnetic resonance imaging (MRI) and gastroduodenoscopy are the other modalities that can be used for diagnosis, CT images were sufficient and MRI was not required in our case. Our not having a diagnostic sign upon endoscopy might have been caused by the fact that the endoscope we used was without lateral vision.

In spite of the diagnostic workup performed before the operation, accurate diagnosis of duodenal duplication is by histological examination. According to the analysis made by Merrot *et al*[1], two types of intra- or juxta-duodenal duplications occur: (1) a common wall formed by two separate mucosae with their own muscularis mucosa and a layer of intervening connective tissue; and (2) a common wall that comprises two mucosal layers with two smooth muscle layers, but that also contains biliary and pancreatic ducts. In our case, histological diagnosis of intraduodenal duplication was made by observation of the mucosa on both sides, each with its own muscularis mucosa, and connective tissue fibers between the two layers.

Management of duodenal duplications depends on the volume, type, location and proximity to the duodenal wall, pancreas or biliary ducts. If there is no communication between the mass and the biliary or pancreatic ducts, and if the vasculature allows, total resection is the procedure of choice. However, if it is not possible, partial resection or internal derivation must be carried out. In partial resection, all of the cyst wall is removed wherever possible, while the area of maximum adherence to the duodenum is preserved[1,13-15]. In our case, duplication was communicating and preservation of duodenal continuity would have been impossible if total resection had been performed. Thus, we performed partial resection with maximal removal of the cystic wall, which allowed secure closure. As the diameter of the communication was not efficient for adequate drainage of the cyst, the common wall between the cyst and the duodenum was excised and the corners were sutured to form a large cystoduodenostomy. This procedure is known as intraduodenally performed internal derivation, and it forms the basis of the endoscopic treatment of duodenal duplication[16].

In conclusion, duodenal duplication should be considered in the differential diagnosis of a patient who presents with abdominal symptoms when cystic structures neighboring the duodenum are demonstrated by radiology. Ideal treatment is total excision but, if not possible, subtotal excision and/or internal derivation should be performed.

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CASE REPORT

Deep venous thrombosis after gastrectomy for gastric carcinoma: A case report

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Abstract

The treatment of gastric carcinoma consists of neoadjuvant chemoradiation, partial gastrectomy, subtotal gastrectomy, total gastrectomy, extended resection, and postoperative chemotherapy. Currently, gastrectomy and extended lymphadenectomy is the optimal choice for late gastric carcinoma. Postoperative complications are common after total gastrectomy including hemorrhage, anastomotic leakage, fistula, and obstruction. However, deep venous thrombosis (DVT) is an uncommon complication after gastrectomy for gastric carcinoma. We describe a case of a 68-year-old female patient with DVT after gastrectomy for gastric carcinoma. The patient was treated with anticoagulants and thrombolytics and subjected to necessary laboratory monitoring. The patient recovered well after treatment and was symptom-free during a 3-mo follow-up. We conclude that correct diagnosis and treatment of DVT are crucial.

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Key words: Gastric carcinoma; Gastrectomy; Deep venous thrombosis; Postoperative complication; Anticoagulant; Thrombolytic therapy; Low molecular weight heparins; Streptokinase; Warfarin sodium

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INTRODUCTION

Deep venous thrombosis (DVT) is an uncommon complication after gastrectomy for gastric carcinoma. DVT may indicate a worse prognosis. Hence, the correct diagnosis and effective methods to prevent and treat DVT are important and can reduce morbidity and mortality of the disease. Low-molecular-weight heparins (LMWHs) play a major role in the management of DVT. We report, in this paper, a case of a 68-year-old female patient with DVT after total gastrectomy for gastric carcinoma, who underwent thrombolytic and anticoagulant therapies successfully.

CASE REPORT

A 68-year-old female patient, complaining of left leg pain and tumefaction, was admitted to our surgical department in May 2007. She underwent total gastrectomy for primary gastric adenocarcinoma 3 mo ago. Physical examination revealed left leg tumefaction and pressure pain below the inguinal triangle. Type B ultrasound (Figure 1A and B) showed left leg DVT. After diagnosis, thrombolytic therapy and anticoagulant therapy were performed, in which streptokinase (600 000 U/d, days 1-3) was infused iv, LMWH (0.4 mL/d, days 1-14) was subcutaneously injected and warfarin sodium (2.5 mg/d, days 1-7) was administered orally. Laboratory monitoring showed both prothrombin time and thrombin time were normal during the thrombosis treatment The patient recovered well after the treatment. Two weeks after treatment, B type ultrasound (Figure 2) showed the partial recirculation of the left leg DVT. The patient was free of symptoms and signs of recurrent DVT during a 3-mo follow-up.

DISCUSSION

The treatment of gastric carcinoma consists of neoadjuvant chemoradiation, partial gastrectomy,

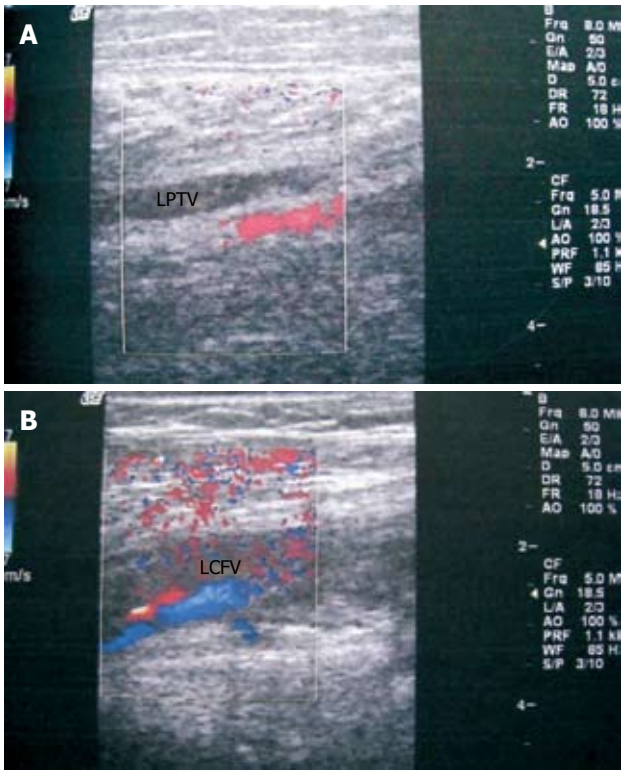

Figure 1 B type ultrasound (A and B) showed the left leg DVT.

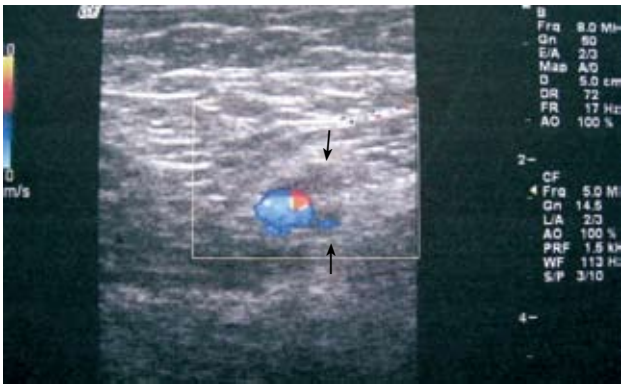

Figure 2 B type ultrasound after treatment showed partial recirculation of the left leg DVT.

subtotal gastrectomy, total gastrectomy, extended resection, and postoperative chemotherapy. Currently, gastrectomy and extended lymphadenectomy is the optimal choice for late gastric carcinoma[1]. Postoperative complications after total gastrectomy included hemorrhage, anastomotic leakage, fistula, and obstruction[2-6]. However, DVT is an uncommon complication after gastrectomy for gastric carcinoma. Venous thromboembolism (VTE), manifested as either DVT or pulmonary embolism, is an extremely common medical problem, occurring either in isolation or as a complication of other diseases or procedures[7,8]. López and Conde discussed the mechanisms and proposed that venous thrombi may be initiated on the vessel wall in the absence of anatomically overt vessel wall injury. Elevations in the levels of TF-bearing microvessels associated with inflammatory conditions would help explain the increased risk of thrombosis associated with infections and inflammatory states such as inflammatory bowel disease. The study provides an algorithm for using risk assessment as a means of determining the length and type of therapy to be used to minimize the recurrence, while diminishing the risk of simultaneous bleeding associated with anticoagulation[7]. Patients with cancer make up approximately 20% of those presenting with first-time VTE, and the presence of VTE anticipates a much poorer prognosis for patients with cancer, probably because of the morbidity associated with VTE itself and because VTE may herald a more aggressive cancer[7,9]. Chemotherapy can increase the risk of venous thrombosis in breast cancer patients. This risk increase appears to be greatest in postmenopausal patients[10]. A hypercoagulative state is observed in cancer patients, as shown by abnormal "routine" blood tests found in up to 90% of these patients, as well as increased levels of specific markers of coagulative activation[9,10].

LMWH is the drug of choice for the prevention and treatment of VTE in patients with cancer[11]. For prophylaxis in the surgical setting, a single dose of subcutaneous LMWH is as effective and safe as multiple doses of unfractionated heparin. Extending prophylaxis with LMWH beyond hospitalization was recently found to reduce the risk of postoperative thrombosis after abdominal surgery for cancer. The potential anti-neoplastic effects of LMWHs make these agents an attractive option for patients with cancer[11]. The study of López indicates that LMWH improves the survival of patients with advanced cancer through mechanisms beyond their effect as anticoagulants. As a result of their improved efficacy and safety and potential anti-neoplastic effect, LMWHs have become the anticoagulants of choice for treating VTE associated with cancer[7-9].

DVT is a severe problem in patients with cancer that complicates the management and predicts a worse prognosis. The pathophysiology of this thrombophilic state is complex due to interactions of tumor cells and their products with host cells. Risk of thrombotic complication can be reduced and survival improved by administration of anti-coagulants[8-10,12]. LMWH has simplified and improved the management of VTE, and recent studies suggest that it may improve the survival of cancer patients. This review provides an update on the primary prevention and treatment of VTE, as well as prophylaxis of central venous catheters in patients with malignancies[9,10].

DVT is a serious complication of gastrectomy and is historically associated with a high mortality. We conclude that correct diagnosis and treatment of DVT after surgery or chemotherapy are crucial.

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CASE REPORT

Signet-ring cell carcinoma of ampulla of Vater: Contrast-enhanced ultrasound findings

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Abstract

Signet-ring cell carcinoma (SRCC) of ampulla of Vater is extremely uncommon, and less than 15 cases have been reported so far in literature. It mainly occurs in elderly people (median age 57 years). We report a rare case of SRCC of the ampulla of Vater in a 38-year-old woman who presented with a small tumor at the Vater, discovered by the contrast-enhanced ultrasound (CEUS). Histopathological examination showed prominent signet-ring features. We also describe the imaging features of SRCC of ampulla of Vater in CEUS.

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Key words: Signet-ring cell carcinoma; Ampulla of Vater; Contrast-enhanced ultrasound

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INTRODUCTION

Signet-ring cell carcinoma (SRCC) usually occurs in the gastrointestinal tract. The World Health Organization (WHO) defines it as a special type or a variant of gastrointestinal adenocarcinoma. SRCs may exist alone or coexist with any other types of malignant gastrointestinal tumors. SRCC is very rarely found among carcinomas of the ampulla of Vater. Here, we describe one patient with SRCC in the ampulla of Vater, which was found by contrast-enhanced ultrasound (CEUS). This is the first case reported in literature, which was successfully diagnosed with CEUS.

CASE REPORT

A 38-year-old woman was hospitalized because of pruritus for 13 d, and dermatic and scleral jaundice with urine the color of bean oil for 5 d. The stool s had a silver color. The patient had nausea but without vomiting, fever and abdominal pain. She lost weight of about 3 kg in 1 mo. She had a history of surgery for left breast adenoma at another institution several years ago.

Physical examination revealed mucocutaneous jaundice without tenderness in the epigastrium. The laboratory test results showed that white blood cells and hemoglobin were normal. Biochemical tests demonstrated the presence of glutamate-pyruvate transaminase at 446.5 IU/L (normal range, 0-40), glutamic-oxal (o) acetic transaminase at 277.3 IU/L (normal range, 5-34), alkaline phosphatase at 744.1 IU/L (normal range, 40-150), γ-glutamyltransferase at 1687.2 IU/L (normal range, 9-64), total bilirubin at 186.6 mg/dL (normal range, 3.4-20.5), direct bilirubin at 154.2 mg/dL (normal range, 0-8.6), and indirect bilirubin at 32.4 mg/dL (normal range, 3.4-11.9). The tumor markers of carcinoembryonic antigen were 4.74 ng/mL (normal range, 0-5), alpha fetoprotein 3.87 ng/mL (normal range, 0-9), and carbohydrate antigen 19-9 143.13 ng/mL (normal range, 0-37). Endoscopic ultrasound (Figure 1) showed a heterogenic, hypoechoic mass with ill-defined margins at the junction of the common bile duct (CBD) and the main pancreatic duct (PMD)- ampulla of Vater.

Conventional gray-scale ultrasound using a Logiq 9 scanner (GE, USA) equipped with a C2-4 transducer with a central frequency of 3.5 MHz revealed that the

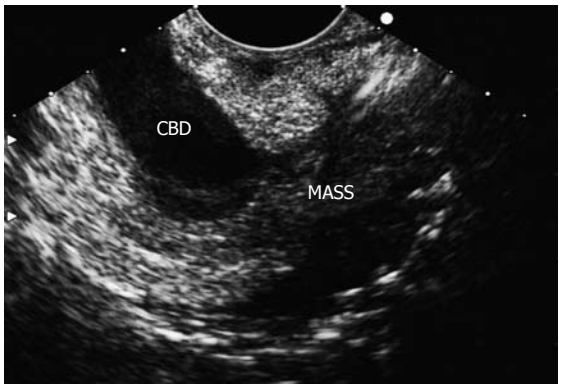

Figure 1 A heterogenic and hypoechoic mass (MASS) with ill-defined margin about 2.7 cm x 1.8 cm at the junction of the CBD and MPD.

Figure 2 The end part of the CBD (arrow), which suddenly became narrow with a diameter of 0.7 cm. There was no exact mass detected.

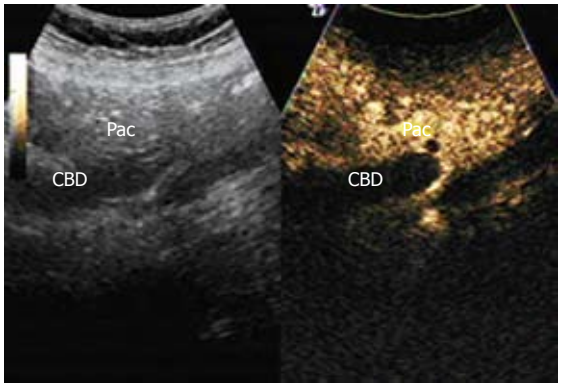

Figure 3 The wall of the CBD enhanced at 12 s after contrast agent was administered, while there was no obvious hyper-enhanced or hypo-enhanced lesion in the ampulla of Vater.

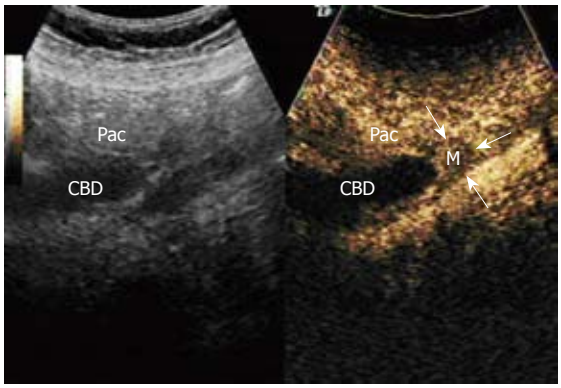

Figure 4 A hypo-enhanced lesion about 1.7 cm x 1.6 cm with blurred borders in the ampulla of Vater, from 20 s to 180 s, in comparison with the adjacent pancreas Pac.

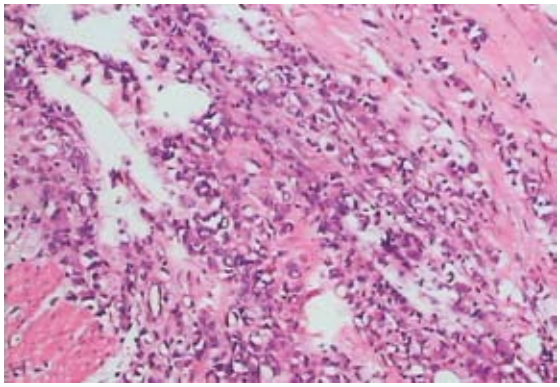

Figure 5 Micrograph shows SRCC of ampulla of Vater (HE x 100).

intrahepatic bile duct was dilated with a diameter of 0.8 cm, and the initial and intermediate portion of the CBD were dilated with a maximal diameter of 2.0 cm. The end part of the CBD suddenly became narrow, with a diameter of 0.7 cm, and there was no exact mass at the end of the CBD (Figure 2). CEUS was performed with low acoustic power, providing real-time imaging using low-mechanical index modes. Contrast-specific CEUS mode of contrast pulse sequencing was applied. The contrast agent, SonoVue (Brocca, Milan, Italy) (2.4 mL) was administered. The wall of the CBD began to enhance at 12 s after contrast agent was administered (Figure 3), while there was no obvious hyper-enhanced or hypo-enhanced lesion in the ampulla of Vater. A hypo-enhanced lesion about 1.7 cm × 1.6 cm with blurred borders in the ampulla of Vater was found from 20 s to 180 s, compared with the adjacent pancreas (Figure 4). At delayed phase (120 s after contrast agent administration), we scanned the whole liver and no abnormal enhanced lesions were found, indicating that there was no metastases in the liver.

The patient underwent a pancreato-duodenectomy with an extended lymphadenectomy and gastrectomy of 1/4 of the normal stomach. The mass was located in the ampulla of Vater with a size about 2.0 cm × 2.0 cm, brittle and protruding to the cavity of the duodenum. It had infiltrated to the periphery pancreatic tissue and adhered to the inferior vena cava. Lymph nodes of No. 16 were tumescent. The final pathological examination (Figure 5) showed that the cancer cells were widespread and polygonal, and the nuclei of the cells were located on one side, which are prominent signet-ring features. Final pathology confirmed an SRCC of the ampulla of Vater, and the pancreas and the whole wall of the duodenum were infiltrated with carcinoma. No distal or nodal metastases were identified. There was no evidence of lymphatic and vascular invasion. The ampullary cancer was

diagnosed as T3N0M0, stage Ⅱ A according to American Joint Committee on Cancer TNM classification[1] and International Union Against Cancer TNM classification[2].

Our patient did not receive adjuvant therapies such as chemotherapy or radiotherapy after the operation. The patient remained well and had no evidence of recurrence and distant metastases during the 6-mo follow-up.

DISCUSSION

SRCC can arise in many organs, but it usually occurs in the gastrointestinal tract, especially in the stomach. It has been reported that 90% of SRCC occurs in the stomach, with the rest arising in several other organs, including the breast, gallbladder, pancreas, urinary bladder and colon[3]. It is extremely uncommon in the ampulla of Vater. Less than 15 cases have been described in the literature. Akatsu[4] has summarized the previous 14 cases (eight men and six women) and concluded that the median age at diagnosis was 57 years (range, 32-83 years), approximately 15 years older than SRCC of the stomach, but similar to the median age for SRCC of the large bowel. Our case was a 38-year-old woman. It is very rare[5]. The origin for SRCC of the ampulla of Vater remains controversial; one theory is that these tumors may originate from heterotopic gastric mucosa. Another theory holds that these carcinomas arise from areas of gastric-type metaplastic epithelia, which are considered to be a protective response to elevated acidity and are observable in the duodenal bulb of peptic ulcer patients[6]. However, there was no history of peptic ulcer disease in our patient and no ectopic gastric epithelium was found in the tumor and peritumoral tissues. Surgery is the the first choice for the treatment of such disease. As for the prognosis, poorly differentiated adenocarcinoma is more frequently associated with an advanced tumor stage and poor prognosis in cases of ampullary carcinoma. SRCC elsewhere in the gastrointestinal tract has a poor prognosis. However, it is unclear whether the prognosis of a patient with SRCC is worse than that of patients with ordinary carcinoma occurring in the ampulla of Vater, because of the small number of cases so far reported. A patient has been reported to survive for 7.5 years after radical resection, and the author has suggested that long-term survival is possible in ampullary SRCC without nodal involvement[4]. A 58-year-old patient lived for 134 mo after resection and had no evidence of recurrence[7].

As for diagnosis of SRCC, helical computed tomography (CT) only showed a dilated CBD without a mass lesion in the ampulla of Vater in some cases[5,7-9]. Upon ultrasound, it may present as an abnormal echoic mass in the ampulla of Vater[6], or obstruction and dilation of the CBD at the level of the pancreas[8,9]. Our case is unique because we underwent a special examination with CEUS before surgery and found the lesion, which was not discovered by conventional gray-scale ultrasound.

CEUS has gained increasing interest in recent years. The properties of SonoVue and the high sensitivity of recent ultrasound equipment to the presence of microbubbles have shown that CEUS is potentially very useful in revealing many organs and vascular structures. It has been a rapidly evolving technique for clinical application. CEUS allows the assessment of the macrovasculature and microvasculature in different parenchymas, and the identification and characterization of lesions in organs. It has been reportes that CEUS produced results very similar to those obtained with contrast-enhanced CT and magnetic resonance imaging in the characterization of various liver lesions. The causes of obstructive jaundice can be divided into two categories: tumorous and non-malignant stenosis. For non-malignant stenosis, such as acute or chronic inflammation of the papilla, fibroid stenosis at the end of the CBD can be irritated by cholesterol calculi or sludge at the end of the CBD; blood clots at the end of the CBD may cause obstructive jaundice too. However, the main cause of obstructive jaundice is the tumors arising from the ampulla of Vater, and mostly are malignant. In the diagnosis of obstructive jaundice, the emphasis should be laid on excluding the non-malignant reasons: non-shadowing stones, blood clots and sludge. This may influence the selection of therapy. The non-shadowing stones, blood clots and sludge may appear non-enhanced by CEUS because of an absence of blood supply. In the present case, the carcinoma showed iso-enhancement at an early stage after contrast agent administration, and obvious hypo-enhancement at the delayed phase, because it had intravital tissue with blood supply; microbubbles are distributed within the blood and appear wherever there is a blood supply. Our case showed that CEUS may provide an effective means of diagnosis of ampullary carcinomas. CEUS could offer real-time imaging of the microcirculation in the lesions. By CEUS, the lesion may be displayed much clearer than by conventional gray-scale ultrasound. It can also offer a good method in the discrimination of ampullary carcinoma from non-malignant lesions.

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Meetings

Events Calendar 2009

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Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

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

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

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

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

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

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

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

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

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

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

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

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

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

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

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

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

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

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

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

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

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Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

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

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

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World Journal of Gastroenterology (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open-access (OA) peer-reviewed journal supported by an editorial board consisting of 1212 experts in gastroenterology and hepatology from 60 countries.

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The major task of *WJG* is to rapidly report the most recent results in basic and clinical research on gastroenterology, hepatology, endoscopy and gastrointestinal surgery fields, specifically including autoimmune, cholestatic and biliary disease, esophageal, gastric and duodenal disorders, cirrhosis and its complications, celiac disease, dyspepsia, gastroesophageal reflux disease, esophageal and stomach cancers, carcinoma of the colon and rectum, gastrointestinal bleeding, gastrointestinal infection, intestinal inflammation, intestinal microflora and immunity, irritable bowel syndrome; liver biology/pathobiology, liver failure, growth and cancer; liver failure/cirrhosis/portal hypertension, liver fibrosis; *Helicobacter pylori*, hepatitis B and C virus, hepatology elsewhere; pancreatic disorders, pancreas and biliary tract disease, pancreatic cancer; transplantation, genetics, epidemiology, microbiology and inflammatory disorders, molecular and cell biology, nutrition; geriatric gastroenterology, pediatric gastroenterology, steatohepatitis and metabolic liver disease; diagnosis and screening, endoscopy, imaging and advanced technology.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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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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Electronic journal (list all authors)

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

Patent (list all authors)

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

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EDITORIAL

Liver disease in pregnancy

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Abstract

Liver diseases in pregnancy may be categorized into liver disorders that occur only in the setting of pregnancy and liver diseases that occur coincidentally with pregnancy. Hyperemesis gravidarum, preeclampsia/eclampsia, syndrome of hemolysis, elevated liver tests and low platelets (HELLP), acute fatty liver of pregnancy, and intrahepatic cholestasis of pregnancy are pregnancy-specific disorders that may cause elevations in liver tests and hepatic dysfunction. Chronic liver diseases, including cholestatic liver disease, autoimmune hepatitis, Wilson disease, and viral hepatitis may also be seen in pregnancy. Management of liver disease in pregnancy requires collaboration between obstetricians and gastroenterologists/hepatologists. Treatment of pregnancy-specific liver disorders usually involves delivery of the fetus and supportive care, whereas management of chronic liver disease in pregnancy is directed toward optimizing control of the liver disorder. Cirrhosis in the setting of pregnancy is less commonly observed but offers unique challenges for patients and practitioners. This article reviews the epidemiology, pathophysiology, diagnosis, and management of liver diseases seen in pregnancy.

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Key words: Liver disease; Pregnancy; Maternal outcome; Fetal outcome; Cesarean section; Cholestasis; Viral hepatitis.

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INTRODUCTION

Liver diseases in pregnancy are usually categorized into liver disorders that occur only in pregnancy and liver diseases that occur coincidentally in pregnancy. There are five liver disorders that are pregnancy-specific: hyperemesis gravidarum, preeclampsia/eclampsia, syndrome of hemolysis, elevated liver tests, and low platelets (HELLP), acute fatty liver of pregnancy, and intrahepatic cholestasis of pregnancy. These disorders typically occur at specific times during the course of pregnancy (Table 1), and they may lead to significant maternal and fetal morbidity and mortality. There is a role for certain medications in these disorders, but the risks and benefits of the use of such therapies must be considered (Table 2). Delivery of the fetus usually terminates the progression of these disorders. Chronic liver diseases that occur coincidentally in pregnancy include cholestatic liver disease, autoimmune hepatitis, Wilson disease, and viral hepatitis. Some of the pharmacological agents used to treat chronic liver disease may be used in pregnancy, but there are other agents whose teratogenicity precludes use in pregnancy. Although uncommon, women with cirrhosis may become pregnant and may have a relatively benign course of pregnancy. However, the presence of portal hypertension may contribute to maternal complications. Given the complexity of these disorders and the potential risks to both the mother and the fetus, it is important that obstetricians and gastroenterologists/hepatologists collaborate in providing management of liver disease in pregnancy.

HYPEREMESIS GRAVIDARUM

Hyperemesis gravidarum (HG) is defined as intractable nausea and vomiting during pregnancy that often leads to fluid and electrolyte imbalance, weight loss of 5% or greater, and nutritional deficiency requiring hospital admission[1]. The incidence of HG varies from 0.3%-2% of all live births[2]. HG often occurs between the 4th and 10th wk of gestation and usually resolves by the 20th wk.

Table 1 Features of pregnancy-associated liver diseases

Disease	Timing of occurrence	Clinical features	Histology
Hyperemesis gravidarum	First trimester	Nausea, vomiting, weight loss, nutritional deficiency	No distinct histopathology, may see normal tissue or hepatocyte necrosis, bile plugs, steatosis
Preeclampsia/eclampsia	Second/third trimester	Hypertension, edema, proteinuria, neurological deficits (headaches, seizures, coma)	Periportal hemorrhage, necrosis, fibrin deposits, may see microvesicular fat
Syndrome of hemolysis, elevated liver tests, and low platelets (HELLP)	Third trimester	Abdominal pain, nausea, vomiting, edema, hypertension, proteinuria	Necrosis, periportal hemorrhage, fibrin deposits
Acute fatty liver of pregnancy (AFLP)	Third trimester	Nausea, vomiting, abdominal pain, fatigue, jaundice	Microvesicular fat
Intrahepatic cholestasis of pregnancy (ICP)	Second/third trimester	Pruritus, jaundice, fatigue, abdominal pain, steatorrhea	Centrilobular cholestasis, no inflammation

Table 2 Safety of drugs used in pregnancy-associated liver diseases

Drug	FDA pregnancy category	Comments
Antiemetics		
Promethazine	C	Possible respiratory depression if drug is administered near time of delivery
Metoclopramide	B	Available evidence suggests safe use during pregnancy
Ondansetron	B	Additional studies are needed to determine safety to the fetus, particularly during the first trimester
Prochlorperazine	C	There are isolated reports of congenital anomalies; however, some included exposures to other drugs. Jaundice, extrapyramidal signs, hyper-/hyporeflexes have been noted in newborns
Antihypertensives		
ACE inhibitors	C/D	First trimester exposure to ACE inhibitors may cause major congenital malformations Second and third trimester use of an ACE inhibitor is associated with oligohydramnios and anuria, hypotension, renal failure, skull hypoplasia, and death in the fetus/neonate
Beta blockers	C/D	Fetal bradycardia, hypotension, risk of intrauterine growth retardation
Calcium channel blockers	C	Teratogenic and embryotoxic effects have been demonstrated in small animals. There are no adequate and well-controlled studies in pregnant women
Anticoagulation		
Aspirin	C (1st/2nd trimesters) D (3rd trimester)	Adverse effects in the fetus include intrauterine growth retardation, salicylate intoxication, bleeding abnormalities, and neonatal acidosis. Use of aspirin close to delivery may cause premature closure of the ductus arteriosus. Data have shown low-dose aspirin (60-150 mg/day) may be safe in pregnancy
Enoxaparin	B	No adequate and well-controlled studies using enoxaparin. Postmarketing reports include congenital abnormalities and also fetal death
Heparin	C	Does not cross the placenta
Intrahepatic cholestasis		
Ursodeoxycholic acid	B	Relatively low risk
S-adenosyl-L-methionine	Not evaluated by FDA	Relatively low risk
Cholestyramine	C	Cholestyramine is not absorbed systemically, but may interfere with vitamin absorption

United States Food and Drug Administration (FDA) pregnancy categories: Category A: Well-controlled studies failed to show a risk to the fetus in the first trimester of pregnancy (and there is no evidence of risk in the second or third trimesters). Category B: Animal reproduction studies failed to show a risk to the fetus, and there are no adequate studies in pregnant women. Category C: Animal reproduction studies have shown an adverse effect on the fetus. There are no adequate studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks. Category D: There is evidence of human fetal risk based on data from investigational or marketing experience or studies in humans. However, the potential benefits may warrant use of the drug in pregnant women despite potential risks. Category X: Data have demonstrated fetal abnormalities in animals and humans, and/or there is positive evidence of human fetal risk based on data from investigational or marketing experience. The risks of the use of the drug in pregnant women outweigh potential benefits.

However, in approximately 10% of HG patients, symptoms continue through pregnancy and resolve only with delivery of the fetus[3].

HG remains a poorly understood condition and most likely involves a combination of hormonal, immunologic, and genetic factors. Data have shown increased levels of human chorionic gonadotropin (HCG) in HG, and proposed mechanisms for the effect of HCG on HG include stimulation of secretory processes of the upper gastrointestinal tract and stimulation of the thyroid gland[4-7]. Other proposed factors contributing to HG include elevations of estrogen, decreases in prolactin levels, and overactivity of the hypothalamic-pituitary-adrenal axis[6]. It has been speculated that immune and inflammatory mechanisms also contribute to HG. In particular, increased levels of tumor necrosis factor-alpha have been observed in HG patients[8]. Higher levels of immunoglobulin G (IgG), immunoglobulin M (IgM), C3, and C4 levels, as well as increased lymphocyte counts and natural killer and extra-thymic T cell levels have been observed in HG patients[9,10].

Liver involvement is seen in about 50%-60% of

patients with HG[11]. Most commonly seen are mild serum aminotransferases elevations, but there are reported cases of severe transaminase elevations (alanine aminotransferase (ALT) levels 400 to over 1000 U/L)[12]. Mild hyperbilirubinemia with mild jaundice can be seen as well. Other complications include disturbances in electrolytes and in water and acid-base balance that can usually be treated adequately with hydration.

While maternal morbidity is well documented, the effects of HG on the fetus are less clear. Some data suggest no differences between fetuses born to mothers with HG and non-HG mothers[13], but other data show increased rates of fetal abnormalities including undescended testicles, hip dysplasia, and Down Syndrome[2]. In one large cohort study, infants of HG mothers were found to have lower birth weights and higher rates of being small for gestational age[14]. However, no significant effect on perinatal survival has been shown.

Treatment of HG is primarily supportive. Patients should avoid triggers that aggravate nausea, and eat small, frequent, low-fat meals. Intravenous fluids, thiamine and folate supplementation, and antiemetic therapy may be administered. Promethazine is a first-line agent, but other medications such as metoclopramide, ondansetron, and steroids have also been used. Enteral feeding is effective, and in severe cases, total parenteral nutrition may be used cautiously.

PREECLAMPSIA/ECLAMPSIA

Preeclampsia is a disorder defined by the triad of hypertension, edema, and proteinuria. It affects about 5%-10% of all pregnant women and usually occurs late in the second trimester or in the third trimester. In preeclampsia, hypertension is defined as having a systolic pressure greater than 140 mmHg and a diastolic pressure greater than 90 mmHg on at least two occasions that are at least 4 to 6 h apart in a previously normotensive patient, and proteinuria is defined as equal to or greater than 300 mg of protein in a 24 h urine collection or 1+ protein or greater on urine dipstick testing of two random urine samples collected at least 4 to 6 h apart[15]. Eclampsia involves all features of preeclampsia and includes neurologic symptoms such as headaches, visual disturbances, and seizures or coma. Risk factors for preeclampsia and eclampsia include nulliparity, extremes of maternal age, insulin resistance, obesity, and infection[15,16]. The pathophysiology of preeclampsia/eclampsia is thought to involve procoagulant and proinflammatory states that create glomerular endotheliosis, increased vascular permeability, and a systemic inflammatory response that results in end-organ damage and hypoperfusion.

Abnormal laboratory values include a 10- to 20-fold elevation in aminotransferases, elevations in alkaline phosphatase levels that exceed those normally observed in pregnancy, and bilirubin elevations of less than 5 mg/dL. Liver histology generally shows hepatic sinusoidal deposition of fibrin along with periportal hemorrhage, liver cell necrosis, and in severe cases, infarction; these changes are likely due to vasoconstriction of hepatic vasculature[17]. Microvesicular fatty infiltration has also been observed in some cases of preeclampsia, suggesting a possible overlap with acute fatty liver of pregnancy[18].

Maternal mortality from preeclampsia/eclampsia is rare in developed countries, but may approach 15%-20% in developed countries[15]. Likewise, the fetal mortality rate is rare, occurring in 1%-2% of births. Maternal and neonatal morbidity may include placental abruption, preterm delivery, fetal growth restriction or maternal renal failure, pulmonary edema, or cerebrovascular accident.

The only effective treatment for preeclampsia is delivery of the fetus and placenta. However, if mild preeclampsia is evident before fetal lung maturity at 36 wk gestation, one may consider expectant management with intensive monitoring. Pharmacological agents used in preeclampsia include antihypertensives such as calcium channel blockers and low-dose aspirin. Magnesium sulfate may be administered if eclampsia develops.

HEMOLYSIS, ELEVATED LIVER TESTS AND LOW PLATELETS

HELLP syndrome is a multisystemic disorder of pregnancy involving hemolysis, elevated liver tests, and low platelets. About 70% of cases occur antenatally, and most cases occur during the last trimester of pregnancy[19]. The pathogenesis of HELLP is thought to involve alterations in platelet activation, increases in proinflammatory cytokines, and segmental vasospasm with vascular endothelial damage. An association with a defect in long-chain 3-hydroxyacyl-coenzyme A dehydrogenase (LCHAD) has also been described, suggesting a possible overlap of HELLP syndrome and acute fatty liver of pregnancy.

Most patients present with right upper quadrant abdominal pain, nausea, vomiting, malaise, and edema with significant weight gain. Less commonly associated conditions include renal failure (with increased uric acid), diabetes insipidus, and antiphospholipid syndrome. Other late findings of HELLP include disseminated intravascular coagulopathy (DIC), pulmonary edema, placental abruption, and retinal detachment. Hypertension and proteinuria may be seen, but in 20% of patients, hypertension is absent[19]. Laboratory findings include hemolysis with increased bilirubin levels (usually less than 5 mg/dL) and lactate dehydrogenase (LDH) levels greater than 600 IU/L, moderately elevated aspartate aminotransferase (AST) and ALT levels (200 IU/L to 700 IU/L), and thrombocytopenia (less than 100 000/mL). In early stages, prothrombin time and activated partial thromboplastin time are normal, but in later phases, DIC may be present with increased levels of fibrin degradation products and D-dimer, and thrombin-antithrombin complexes. The pathogenesis

of hepatic damage in HELLP syndrome involves intravascular fibrin deposition and sinusoidal obstruction that can lead to hepatic hemorrhage and infarction. Histologically, one may see focal hepatocyte necrosis, periportal hemorrhage, and fibrin deposits.

The reported maternal mortality from HELLP is 1%, and the perinatal mortality rate ranges from 7%-22% and may be due to premature detachment of placenta, intrauterine asphyxia, and prematurity[11]. Other complications of HELLP syndrome include acute renal failure, adult respiratory distress syndrome, pulmonary edema, stroke, liver failure, and hepatic infarction. The only definitive treatment for HELLP syndrome is delivery. If the pregnant woman is greater than 34 wk gestation, immediate induction is recommended. If gestational age is between 24 wk and 34 wk, corticosteroids are administered to accelerate fetal lung maturity in preparation for delivery 48 h later. After delivery, close monitoring of the mother should continue, as data have shown worsening thrombocytopenia and increasing LDH levels up to 48 h postpartum[20]. However, most laboratory values (transaminases, bilirubin, LDH) normalize in 48 h, and the presence of persistent or worsening laboratory abnormalities by the fourth postpartum day may signal postpartum complications[21]. For patients with ongoing or newly developing postpartum symptoms of HELLP, modalities such as antithrombotic agents, plasmapheresis, and dialysis may be employed.

ACUTE FATTY LIVER OF PREGNANCY

Acute fatty liver of pregnancy (AFLP) is a rare but serious maternal illness that occurs in the third trimester of pregnancy. With an incidence of 1 in 10000 to 1 in 15000 pregnancies, it has a maternal mortality rate of 18% and a fetal mortality rate of 23%[17,22]. AFLP is more commonly seen in nulliparous women and with multiple gestation.

The pathophysiology of AFLP involves defects in mitochondrial fatty acid beta-oxidation. Under normal circumstances, an individual that is heterozygous for enzymatic mutations in fatty acid oxidation will not have abnormal fatty oxidation. However, when a heterozygous woman has a fetus that is homozygous for such mutations, fetal fatty acids accumulate and return to the mother's circulation. The extra load of long-chain fatty acids and subsequent triglyceride accumulation lead to hepatic fat deposition and impaired hepatic function in the mother. A deficiency in long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) is thought to be associated with the development of AFLP. LCHAD is a component of an enzyme complex known as the mitochondrial trifunctional protein (MTP), and it is believed that the G1528C and E474Q mutations of the MTP are responsible for causing LCHAD deficiency that subsequently leads to AFLP[23].

Patients with AFLP typically present with a 1 to 2 wk history of nausea, vomiting, abdominal pain, and fatigue. Jaundice occurs frequently, and some women experience moderate to severe hypoglycemia, hepatic encephalopathy, and coagulopathy. Approximately 50% of these patients will also have signs of preeclampsia, although hypertension is generally not severe[24]. Laboratory findings include elevations in aminotransferase levels, which may range from being mildly elevated to approaching 1000 IU/L. Many cases involve neutrophilic leukocytosis, and as the disease progresses, thrombocytopenia (with or without DIC) and hypoalbuminemia may occur. Rising uric acid levels and impaired renal function may also be seen.

Since AFLP can lead to significant maternal and fetal morbidity and mortality, prompt diagnosis must be made. The most definitive test is liver biopsy. Histopathologic findings reveal swollen, pale hepatocytes in the central zones with microvesicular fatty infiltration that can be identified on frozen section with oil red O staining. Electron microscopy may also show megamitochondria and paracrystalline mitochondrial inclusions. Although liver biopsy may be helpful, it is often not done due to the presence of coagulopathy. Imaging studies, including ultrasound and computed tomography (CT), are inconsistent in detecting fatty infiltration[25,26]. Therefore, the diagnosis of AFLP is usually made on clinical and laboratory findings.

As with most pregnancy-associated liver diseases, the treatment of AFLP involves delivery of the fetus. However, many laboratory abnormalities may persist after delivery and may initially worsen during the first postpartum week. In rare cases, patients will progress to fulminant hepatic failure with need for liver transplantation[27]. In addition to monitoring the mother closely, careful attention should also be paid to the infant given the increased risk of cardiomyopathy, neuropathy, myopathy, nonketotic hypoglycemia, hepatic failure, and death associated with fatty acid oxidation defects in newborns. Finally, affected patients should be screened for defects in fatty acid oxidation as recurrence in subsequent children is 25%, and recurrence of AFLP in mothers is also possible[11,23].

INTRAHEPATIC CHOLESTASIS OF PREGNANCY

Intrahepatic cholestasis of pregnancy (ICP), also known as obstetric cholestasis, is a rare pregnancy-specific liver condition that occurs in the late second or third trimester and has a prevalence of about 1/1000 to 1/10000. It is significantly more common in South Asia, South America (especially Chile), and Scandinavian countries. ICP is also more common in women of advanced maternal age, multiparous women, and in women with a personal history of cholestasis with oral contraceptive use[28]. The prognosis for women with ICP is usually good, but it is associated with increased fetal morbidity and mortality, particularly from chronic placental insufficiency, preterm labor, fetal distress, and intrauterine death[29].

The etiology of ICP is likely multifactorial and

may include genetic, hormonal and environmental variations. Mutations in the phospholipid translocator known as the ATP-cassette transporter B4 (ABCB4) or multidrug resistant protein-3 (MDR3) are associated with the development of ICP[30]. Changes induced by these genetic mutations lead to increased sensitivity to estrogen, which impairs the sulfation and transportation of bile acids. The pregnancy-associated increase in estrogen may also contribute to ICP. This is supported by the fact that women with multiple gestations and proportional increases in estrogens have an increased risk of ICP[31]. Estrogens are thought to act on hepatocytes by decreasing membrane permeability and bile acid uptake by the liver. The maternal-to-fetal transfer of bile acids across the placenta becomes impaired, leading to potentially toxic bile acid levels in the fetus[32]. The elevation in bile acid levels is also thought possibly to affect myometrial contractility and to cause vasoconstriction of chorionic veins in the placenta, which may contribute to preterm deliveries and fetal distress seen in ICP[33,34].

Maternal complications are much less severe. The classic symptom is pruritus that usually begins in the second or third trimester. It usually occurs in the palms and soles and may progress to the rest of the body, and the pruritus is often worse at night. Pruritus may be severe but is usually relieved within 48 h after delivery of the fetus. Jaundice occurs in approximately 10%-25% of patients and may appear within the first four weeks of the onset of pruritus[35]. Cholelithiasis and cholecystitis have been observed to occur with greater frequency in women with ICP[36]. Other symptoms include fatigue, anorexia, epigastric pain, and steatorrhea due to fat malabsorption. Malabsorption may also lead to vitamin K deficiency leading to prolonged prothrombin times and postpartum hemorrhage.

Abnormal laboratory findings include elevated total bile acid levels up to 10- to 25-fold, with an increase in cholic acid and a decrease in chenodeoxycholic acid leading to a marked elevation in the cholic/chenodeoxycholic acid ratio. The glycine/taurine ratio is also reduced. Other findings include mild aminotransferase elevations, which are seen in about 60% of ICP patients. AST and ALT levels rarely exceed two times the upper limits of normal, but may approach 10- to 20-fold elevations in rare cases. Bilirubin levels may be elevated, but are usually less than 6 mg/dL. Serum alkaline phosphatase levels may also be elevated, but this is usually less helpful to follow given typical alkaline phosphatase elevations seen in pregnancy. Histopathologic findings on liver biopsy include nondiagnostic centrilobular cholestasis without inflammation and bile plugs in hepatocytes and canaliculi[17]. Liver biopsy is usually not required to make the diagnosis of ICP.

The treatment of choice for ICP is ursodeoxycholic acid (UDCA), which helps to relieve pruritus and improve liver test abnormalities. It is unclear how UDCA works, but it is felt that UDCA conjugates help target and insert key transporter proteins, such as MRP2 (ABCC2) or bile salt export pumps (ABCB11) into the canalicular membranes[37]. Data have also shown that UDCA increases expression of placental bile acid transporters, which may allow for improved bile acid transfer[38]. Other medications, such as cholestyramine and S-adenosyl-L-methionine, have been associated with improving pruritus and normalizing biochemical profiles, but studies have found UCDA to be superior over cholestyramine and S-adenosyl-L-methionine[39,40]. Dexamethasone has also been used, but has shown to be much less effective in reducing bile acids and bilirubin and ineffective in relieving pruritus[41]. Antihistamines are frequently used to alleviate pruritus, and vitamin K and other fat-soluble vitamin supplementation should also be administered if fat malabsorption is suspected.

GALLSTONES

The formation of biliary sludge and gallstones is associated with parity. The prevalence of gallstones in pregnancy is 18.4%-19.3% in multiparous women and 6.9%-8.4% in nulliparous women[42]. The etiology for an increased prevalence of biliary sludge and gallstones in pregnancy is multifactorial. Increased estrogen levels, especially in the second and third trimesters, lead to increased cholesterol secretion and supersaturation of bile, and increased progesterone levels cause a decrease in small intestinal motility[43]. Also, fasting and postprandial gallbladder volumes are larger, and emptying time is reduced[44]. The large residual volume of supersaturated bile in the pregnant woman leads to biliary sludge and the formation of gallstones. Pre-pregnancy factors observed to be associated with the development of gallstones in pregnancy include a high body mass index, high serum leptin levels, low high-density lipoprotein (HDL) levels, and insulin resistance[45,46].

Pregnant women with gallstones may present with right upper quadrant pain that may radiate to the flank, scapula, or shoulder. They may also report nausea, vomiting, anorexia, fatty food intolerance, and low-grade fever. Conservative medical management is recommended initially, especially during the first and third trimesters, in which surgical intervention may confer risk of abortion or premature labor, respectively. Medical management involves intravenous fluids, correction of electrolytes, bowel rest, pain management, and broad spectrum antibiotics. However, relapse rates (40%-90%) are high during pregnancy; thus, surgical intervention may be warranted[47,48]. Laparascopic cholecystectomy in the second trimester is preferred[49]. Endoscopic retrograde cholangiopancreatography (ERCP) may also be required if there are concerns about choledocholithiasis, and this can be performed safely in pregnancy by shielding the fetus and minimizing fluoroscopy time[50].

PRIMARY BILIARY CIRRHOSIS

Primary biliary cirrhosis (PBC) is a chronic cholestatic disease that affects persons in their 30s to 60s[51]. It is

characterized by progressive destruction of intrahepatic bile ducts and is likely autoimmune in origin, as more than two thirds of patients with PBC have an associated autoimmune disease. The course of PBC may be insidious, often presenting with fatigue and pruritus. Serum aminotransferase, bilirubin, cholesterol, IgM, and erythrocyte sedimentation rate levels are often elevated, and an elevated bilirubin level often portends poor prognosis. Portal hypertension and liver failure may develop[52].

Early reports have suggested that PBC is associated with reduced fertility, amenorrhea, repeated pregnancy loss, endometriosis, and premature ovarian failure, as well as worsening liver function during the course of pregnancy[53-55]. However, more recent data suggest that women with PBC may be able to have normal pregnancies. One study of nine pregnancies in six patients with UDCA-treated PBC showed that all women remained asymptomatic during pregnancy with no recurrence of pruritus[56]. Improvements were seen in laboratory tests including antimitochondrial antibody titers and levels of alkaline phosphatase, ALT, serum bile acid, bilirubin, immunoglobulin G, and immunoglobulin M. However, a flare in disease with increases in liver biochemistries was observed 3 mo postpartum. UDCA has been shown to be safe in pregnancy[56].

PRIMARY SCLEROSING CHOLANGITIS

Primary sclerosing cholangitis (PSC) is a chronic cholestatic syndrome characterized by inflammation, fibrosis, and destruction of intrahepatic and extrahepatic biliary ducts[57]. Though the course is typically variable, PSC is often progressive and leads to biliary cirrhosis. There is no known effective therapy, and liver transplantation is the only option for patients with end-stage PSC. There are only a few published case reports on PSC in pregnancy; thus, the natural history of PSC in pregnancy is not well understood[58-61]. Pregnant patients with PSC may experience pruritus, and complications include biliary strictures and choledocholithiasis. If a patient with PSC develops symptoms worrisome for biliary obstruction, an ultrasound should be performed, as it is thought to be safe in pregnancy and may detect the presence of stones or dominant strictures[61]. Endoscopic retrograde cholangiopancreatography (ERCP) may be considered with caution regarding exposure to radiation and the use of sedation. Empiric use of UDCA should be considered, as it is felt to be safe in pregnancy and improves outcomes of both maternal symptoms and fetal complications[61].

AUTOIMMUNE HEPATITIS

Autoimmune hepatitis (AIH) is characterized by progressive hepatic parenchymal destruction that may lead to cirrhosis. The natural history of AIH in pregnant women is not fully understood, but is thought to be variable. Candia *et al*[62] reviewed 101 cases of AIH in pregnant women reported in the literature between 1966 and 2004 and found that 47 women experienced AIH flares, with 35 occurring during pregnancy and 12 occurring after delivery. Fetal deaths occurred in 19% of pregnancies, and the majority of the fetal deaths occurred before the 20th wk of gestation. However, a more recent review involving a smaller case series of 42 pregnancies in women with AIH reported a fetal loss rate as high as 24%[63]. Fetal death in pregnant women with AIH has been associated with the presence of prematurity and low birth weight[62]. Possible etiologic factors thought to be associated with worsening of AIH in pregnancy include changes in the relative concentrations of various hormones during pregnancy and the presence of specific autoantibodies, including antibodies to SLA/LP and Ro/SSA[63,64].

Pregnant women with AIH are often treated with a combination of steroids and azathioprine. While steroids are thought to be safe in pregnancy, there has been controversy over the use of azathioprine, as earlier studies have shown azathioprine to have teratogenic effects in mice and rabbits[65,66]. It is known that azathioprine crosses the placenta, but more recent data have suggested that azathioprine and its metabolites do not have toxic effects on the fetus[67,68].

Women of childbearing age with AIH should be advised to consider pregnancy only if their disease is well-controlled. However, patients must be monitored closely throughout pregnancy and in the early postpartum period given the unpredictability of the course of AIH in the setting of pregnancy.

WILSON DISEASE

Wilson disease (WD) is a multisystem autosomal recessive disorder of copper metabolism. Occurring in 1:30 000 to 1:50 000 persons, this rare disorder is due to a mutation of the gene, ATP7B, which is located on chromosome 13q14. ATP7B codes for a P type ATPase that controls copper transportation in the liver[69], and more than 100 forms of this mutation have been found to be responsible for the development of WD. This mutation leads to copper excess and deposition in the liver and brain. Hepatic disease may present as chronic hepatitis, cirrhosis, or fulminant hepatic failure; neurologic abnormalities occur in 40%-50% and may include an akinetic-rigid tremor similar to Parkinson's disease, tremor, ataxia, and a dystonic syndrome[70].

Studies on the effect of WD on pregnancy are limited to small case series. It has been proposed that WD may adversely affect fertility due to hormonal fluctuations that can result in amenorrhea; it may also lead to copper deposition in the uterus, resulting in miscarriage due to improper implantation of the embryo[71,72]. Sinha *et al*[73] observed a higher rate of recurrent spontaneous abortions among women with WD who were untreated compared to women with WD who underwent treatment.

Penicillamine, trientine, and zinc are drugs approved by the United States Food and Drug Administration (FDA) as treatment for WD. Penicillamine acts by

reducing chelation and enabling excretion of copper in the urine. Trientine works similarly but is less effective than penicillamine. Zinc induces intestinal cell metallothionein that binds to copper and prevents transfer of copper into the blood. Penicillamine has been reported to cause teratogenicity in animals and humans[74-77]. There is one report of a chromosomal abnormality occurring in a baby delivered by a woman with WD who took trientine during pregnancy, but trientine is known to be teratogenic in animals[78,79]. Brewer *et al*[80] reported that the use of zinc in 26 pregnancies of 19 pregnant women with WD resulted in 24 healthy pregnancies; one baby was born with a heart defect requiring surgery at 6 mo, and a second baby was born with microcephaly.

HEPATITIS B

It is estimated that there are about 350 million chronic carriers of hepatitis B virus (HBV) infection[81]. Perinatal infection is the predominant mode of transmission. Approximately 10%-20% of neonates born to hepatitis B surface antigen (HBsAg)-positive mothers and 90% of those born to both HBsAg- and hepatitis B e antigen (HBeAg)-positive mothers will become infected with HBV[82]. HBV infection early in life usually results in chronic infection, and 25% of these infected persons will die prematurely from cirrhosis and liver cancer[83]. Thus, prevention of vertical transmission is critical.

Immunization with hepatitis B immunoglobulin (HBIG) and hepatitis B vaccine at birth can reduce HBV transmission to less than 10% among infants of mothers who are positive for both HBsAg and HBeAg with even less transmission if the mother is HBeAg negative[84]. All infants born to HBsAg-positive mothers should receive a single hepatitis B vaccine and HBIG (0.5 mL) no later than 12 h after birth, and the hepatitis B vaccination series should be completed, with the second vaccination at one or two months of age and the third vaccination at 6 mo of age[85]. Post-vaccination testing for HBsAg and hepatitis B surface antibody (anti-HBs) should be performed after the complete series of vaccinations at 9 to 18 mo of age in infants born to mothers who are HBsAg positive[86]. It is thought that administration of HBIG and the hepatitis B vaccine within 12 h after birth is 85%-95% effective, and post-birth administration of the hepatitis B vaccination alone is 70%-95% effective in preventing HBV transmission[87].

Data have also shown that use of lamivudine in the last month of pregnancy in HBsAg-positive women may lead to decreased HBV transmission rates, and it has been shown to be safe for use in the last trimester of pregnancy despite its FDA designation as a category C drug[88,89]. Breastfeeding appears not to confer an increased risk of HBV transmission; thus, breastfeeding is not contraindicated in infants of HBsAg mothers[90].

HEPATITIS C

The prevalence of hepatitis C (HCV) in pregnant women in the United States ranges between 1%-2% but may be as high as 4% in some inner-city populations[91]. HCV infection in pregnancy has a presentation that is similar to that of HCV infection in non-pregnant patients. Reports regarding the risk of obstetrical complications among pregnant women infected with HCV are varied. One large cohort study of 506 HCV-positive pregnant women found that HCV infection was associated with the development of gestational diabetes mellitus, lower birth weight, lower Apgar scores, and more admissions to the neonatal intensive care unit for respiratory problems, prematurity, and infections[92]. However, in another study looking at the long term outcomes of 36 women in Ireland inadvertently infected with HCV after exposure to contaminated anti-D immunoglobulin, there were no differences in the rates of spontaneous miscarriage, or birth weights between the HCV-infected group and controls[93].

HCV-infected women do not need to be advised against pregnancy, but they should be counseled on the risks of mother-to-infant transmission of HCV. The risk for vertical transmission of HCV is about 5%-10%. The risk of perinatal transmission of HCV is associated with the presence of HCV RNA in maternal blood at the time of birth and coinfection with human immunodeficiency virus (HIV)[91]. HIV coinfection in pregnant women increases the risk of perinatal HCV transmission by 2-fold, and in more than 25% of cases, both HCV and HIV are transmitted together. Prolonged rupture of membranes (greater than 6 h) has also been associated with an increased risk of perinatal HCV transmission; thus, it is advised that the second stage of labor be kept short in HCV-infected pregnant women[94]. Data on the effects of the mode of delivery on HCV transmission are conflicting; therefore, there are no recommendations regarding the method of delivery that should be used in HCV-infected pregnant women.

Although HCV is detectable in breast milk, there is little documented evidence of transmission of HCV *via* breastfeeding. However, the Centers for Disease Control and Prevention (CDC) recommend that HCV-infected women with cracked or bleeding nipples should abstain from breastfeeding[95].

Combination antiviral therapy with pegylated interferon and ribavirin is generally recommended for HCV-infected patients who are eligible for therapy. However, ribavirin has a category X designation by the FDA as it has been shown to be teratogenic and embryocidal in animal models. Interferon has a designation as category C, as it has been shown to have abortifacient effects in animal models, and there are no adequate studies of its use in pregnant women. Therefore, combination antiviral therapy is not recommended for HCV-infected pregnant women. There are a few reports of women becoming pregnant while on interferon monotherapy for HCV, and in these cases, healthy babies were delivered and were found to have normal growth and development at follow up[96-98]. However, given the uncertainty about safety during pregnancy, it is still recommended that interferon be

avoided by HCV-infected women who are attempting to conceive or are already pregnant.

CIRRHOSIS

Fertility is decreased in women with significant hepatic dysfunction due to hypothalamic-pituitary dysfunction. However, cirrhosis is not a contraindication, as pregnancy may be tolerated if cirrhosis is well-compensated and without features of portal hypertension[99]. Portal hypertension leads to increased maternal complications, including variceal hemorrhage, hepatic failure, encephalopathy, jaundice, malnutrition, and splenic artery aneurysm[100]. Bleeding from esophageal varices has been reported in 20%-25% of pregnant women with cirrhosis[101]. All pregnant women with cirrhosis should be screened for varices starting in the second trimester and started on beta-blockers if indicated. The treatment of variceal bleeding consists of both endoscopic and pharmacologic treatment. However, vasopressin has been shown to cause placental ischemia, necrosis, and amputation of fetal digits and is contraindicated in pregnancy; there is a paucity of information about the use of octreotide in pregnancy[102]. Finally, though there are no good studies evaluating the impact of vaginal delivery of the risk of variceal bleeding, it is recommended that patients have cesarean section to avoid increased straining[103].

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REVIEW

Clinical application of hepatic CT perfusion

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Abstract

Complicated changes occur in hemodynamics of hepatic artery and vein, and portal vein under various kinds of pathologic status because of distinct double hepatic blood supply. This article reviews the clinical application of hepatic computed tomography perfusion in some liver diseases.

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Key words: X-ray computed tomography; Perfusion; Liver; Hemodynamics

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INTRODUCTION

Since Miles *et al*[1] first described liver perfusion computed tomography (CT) imaging (CTP). It has been generally used with the development of imaging techniques and post-processing software. People are no longer satisfied with the diagnosis of diseases due its functional imaging features. Demands of therapeutic effect evaluation, prognosis analysis, *etc*, are rapidly increasing.

PERFUSION PARAMETERS OF NORMAL LIVER

There are some disagreements in normal liver perfusion parameters from different sources due to different choices of computational model and people (Table 1). However, they are all verged about 1/4-1/3 as the ratio of HAP/PVP, in rough approximation of the ratio of blood supply from hepatic artery and portal vein. Pseudo-color images can also be obtained from CT workstation (Figure 1).

PERFUSION IMAGING OF CHRONIC LIVER DISEASES

Chronic liver diseases include chronic hepatitis, liver fibrosis and cirrhosis. Previous studies used liver biopsy as gold standard to assess the degree of liver cirrhosis[6,7]. CTP can show pathological changes in the liver before cirrhosis, even at the early stage of fibrosis, and it can make non-invasive assessment of the degree of chronic liver disease (Table 2).

Guan *et al*[7] induced liver diffuse lesions in rats with diethylnitrosamine. They divided the processes of hepatic diffuse lesions into three stages of hepatitis, hepatic fibrosis, and cirrhosis. In the test group, hepatic arterial perfusion index (HPI) tended to increase gradually, mean transit time (MTT) prolonged obviously, hepatic blood volume (HBV) and hepatic blood flow (HBF) decreased at the same time. The results are consistent with the gradually increased portal vein pressure due to pathological changes.

Van Beers *et al*[8] reported that the total liver perfusion (TLP) is decreased in patients with cirrhosis and non-cirrhotic chronic liver disease, while HPI and MTT are increased. The severity of liver disease, which was categorized into five classes (normal, non-cirrhotic liver disease, Child A, Child B, Child C), was correlated significantly with TLP, HPI, and MTT. The best cutoff point to differentiate patients with cirrhosis from those without cirrhosis was considered a MTT of 22.6 s, with a sensitivity of 81% and a specificity of 81%, respectively.

Hashimoto *et al*[6] reported that HBF decreases with the severity of chronic liver disease. The HPI of patients without liver disease was significantly lower than that of those with Child B and C liver disease. However, the HBV and MTT did not show any correlation between the groups. At the same time, the HPI was correlated

Table 1 Perfusion parameters of normal liver from different sources

Sources (*n*)	HAP	PVP	HPI	HBF	HBV	MTT
Miles *et al*[2] (5)	0.17	0.34	32%			
Blomley *et al*[3]	0.19[1]	0.93[2]				
Weidekamm *et al*[4] (24)	0.2 ± 0.08	1.02 ± 0.35				
Zhou *et al*[5] (4)	0.17 ± 0.08	0.9 ± 0.04	16 ± 16%	106.24 ± 54.53	20.24 ± 8.26	15.06 ± 8.94
Hashimoto *et al*[6] (10)			18.4 ± 5.6%	103.9 ± 18	12.5 ± 2.0	11.1 ± 1.6

[1]*n* = 31, [2]*n* = 19; HAP: Hepatic arterial perfusion (mL/min per millilitre); PVP: Portal vein perfusion (mL/min per millilitre); HPI: Hepatic arterial perfusion index (%); HBF: Hepatic blood flow (mL/min per 100 g); HBV: Hepatic blood volume (mL/100 g); MTT: Mean transit time (s).

Table 2 Changes of perfusion parameters in some liver diseases

Liver disease	Sources	Object	*n*	HAP	PVP	HPI	HBF	HBV	MTT
Chronic	Guan *et al*[7]	Rat	14			↑	↓	↓	↑
Liver	Van Beers *et al*[8]	Patient	34			↑			↑
Disease	Hashimoto *et al*[6]	Patient	38			↑			
Primary	Zhou *et al*[5]	Patient	62	↑		↑	↑		
Hepatic	Tsushima *et al*[11]	Patient	11	↑					
Carcinoma	Komemushi *et al*[12]	Patient	37				↑	↑	
	Sahani *et al*[13]	Patient	25				↑	↑	↓
Liver	Miles *et al*[2]	Patient	4	↑		↑			
Metastatic	Tsushima *et al*[11]	Patient	17	↑					
Disease	Leggett *et al*[16]	Patient	20	↑					
	Shi *et al*[18]	Rat	19	↑	↓				

↑: Increased; ↓: Decreased.

significantly with the degree of fibrosis.

Different study parameters may have a certain discrepancy. Selection of patients, scan parameters and other factors may cause inconsistency.

Li *et al*[9] induced early, intermediate, and advanced stages of liver cirrhosis in rats, while healthy rats received CT hepatic perfusion. At the same time, free portal pressure (FPP) measured with a multiplying channel instrument was closely related with portal vein pressure (PVP), suggesting that CT perfusion is a new non-invasive and efficient modality for the assessment of portal pressure in patients with liver cirrhosis at various stages.

PERFUSION IMAGING IN DETECTING PRIMARY HEPATIC CARCINOMA

CT angiography (CTA) and CT arterial portography (CTAP) can be used to detect the blood flow of hepatic artery and portal vein to study the changes in HCC hemodynamics. Tajima *et al*[10] used CTA and CTAP to detect hepatic nodus confirmed by biopsy. By comparing the ratio of blood vessels in different degrees of dysplasia nodus, HCC and liver parenchyma, they found that the blood flow in artery is increased while that in portal vein is decreased in the nodus. The CTP has gradually substituted the CTA and CTAP in the study of HCC hemodynamics (Figure 2).

Miles *et al*[2] reported that HAP is obviously increased in perimeter of liver tumors, but decreased in the center zone of necrosis. Zhou *et al*[5] showed that the HBF and HPI are higher in primary hepatic carcinoma than in normal liver. The HAP, acquired by analog computation using HBF and HPI, is also higher in primary hepatic carcinoma than in normal liver. Tsushima *et al*[11] showed that the HAP is significantly increased in HCC (0.94 ± 0.26 mL/min per millilitre). Analysis of region of interest (ROI) showed no perfusion of portal vein in the tumor. After transcatheter arterial embolization (TAE), arterial perfusion of tumors was significantly decreased. Komemushi *et al*[12] obtained the pure arterial perfusion of tumor and liver parenchyma by hepatic arteriography, and found that increased arterial BF and BV in tumors are significantly tumors are significantly correlated with rich blood supply in the tumor, revealing that blood-supply in primary hepatic carcinoma is mainly provided by hepatic artery (Table 2).

Sahani *et al*[13] studied perfusion changes in progressive HCC and background liver tissue, showing that the HBF, HBV and permeability-surface area product (PS) are higher in HCC tissue than in background liver tissue, while MTT is decreased. No significant difference was observed in tumor perfusion in the presence and absence of portal vein invasion, confirming that blood supply of primary hepatic carcinoma is mainly provided by hepatic artery.

Changes in perfusion parameters are valuable in qualitative and differential diagnosis of primary hepatic carcinoma. CTP can detect the abnormality of liver perfusion before morphologic change occurs. Fournier *et al*[14] induced primary hepatic carcinoma in a rat model by chemistry, and performed CTP after 11 and 18 wk, respectively, showing that the induced hepatic carcinoma has a high arterial blood flow and a low portal blood flow, with a sensitivity of 87% and a specificity of 80% at week 18, a sensitivity of 86% and a specificity of 65% at week

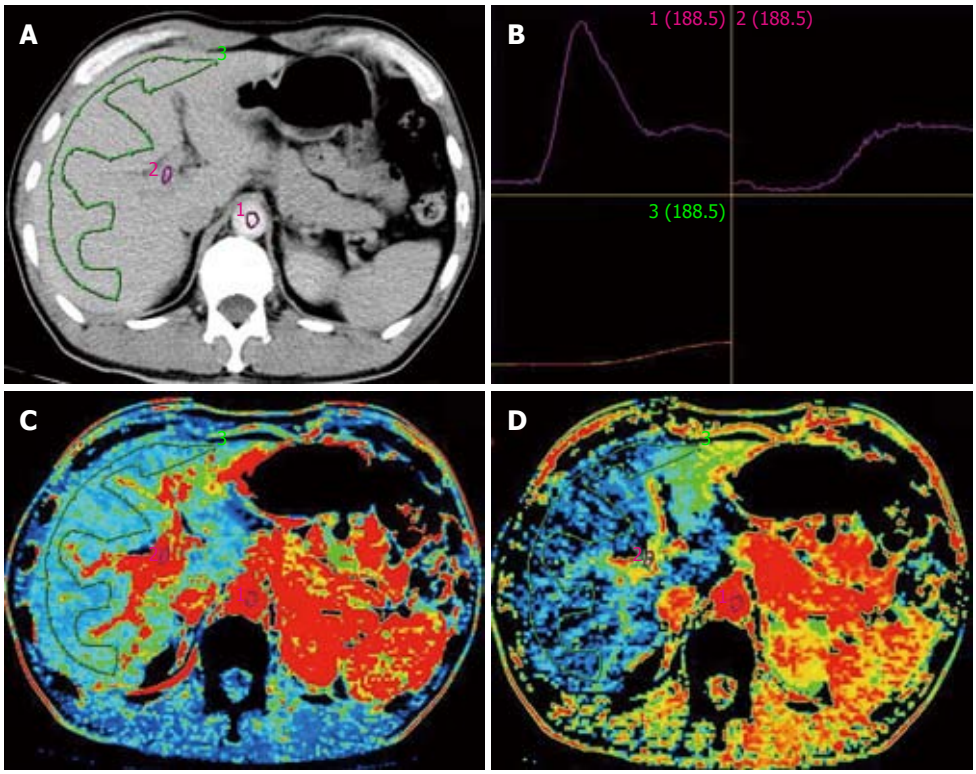

Figure 1 Normal liver perfusion image. A: Selection of ROI at aorta, portal vein and liver; B: Time-density curve (TDC) for ROI (up left: aorta; up right: portal vein; down left: liver); C: Pseudo-color image of HBF; D: Pseudo-color image of HPI.

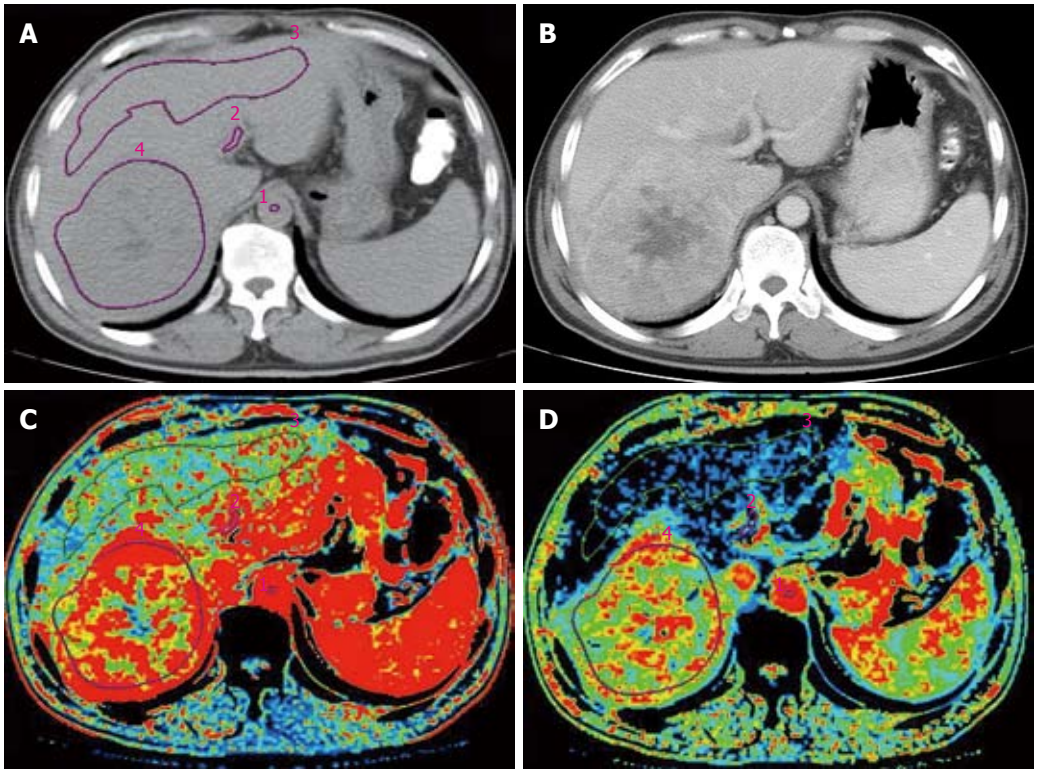

Figure 2 Liver perfusion image of primary hepatic carcinoma at right posterior lobe. A: CT plain image with ROI; B: CT enhanced image of portal vein phase; C: Pseudo-color image of HBF; D: Pseudo-color image of HPI.

11, respectively. The data indicate that CTP can detect hepatic carcinoma at its early stage.

In addition, CTP can evaluate tumor *in vivo*. Tumor tissues with the highest malignant grade can be taken by biopsy under CT guidance, thus avoiding errors of grading occurred in selection of biopsy region.

PERFUSION IMAGING FOR METASTATIC DISEASE

Diagnosis of liver metastatic disease is very important for its staging, prognosis and treatment.

CT perfusion imaging can show the increased arterial perfusion in patients with liver metastatic disease with the slope-ratio analytic methods[2]. Tsushima *et al*[11] showed that HAP of liver metastasis from colon carcinoma is 0.67 ± 0.33 mL/min per millilitre, while that of metastasis from other carcinomas is fluctuant. In a study by Shi *et al*[15], 20 patients with liver metastatic tumors received CTAP via SMA and multi-slice spiral CT perfusion imaging, and found that no tumor vessel can be found in the lesions at portal venous phase of SMA-digital subtraction angiography (DSA). The BF, BV and PS in portal vein in metastatic lesions were lower than those in normal liver tissues, showing that portal vein is not involved in blood supply of liver metastatic tumors (Table 2).

Leggett *et al*[16] also reported that HAP is increased (> 0.25 mL/min per millilitre) in patients with overt colorectal metastasis examined by CTP with slope-ratio analytic methods. PVP showed no statistical variance. However, PVP was decreased in 3 patients as metastasis progressed, indicating that the threshold value of HAP (> 0.25 mL/min per millilitre) can be used to diagnose occult metastasis with a sensitivity of 82%, and no metastasis with a sensitivity of 38%, respectively. Decreased PVP may prognosticate the progression of disease.

Routine CT and MRI are insensitive to occult and early stage hepatic micro metastasis of tumors. Although there is no apparent abnormality in morphology, CTP can display changes in hemodynamics through its functional imaging. Both of increased HAP and HPI can declare the possibility of liver micro metastasis. Cuenod *et al*[17] used the deconvolution method to study liver hemodynamic changes caused by occult hepatic micro metastasis in rat, and found micro metastases in normal liver leads to a 34% decrease in portal blood flow and a 25% increase in MTT, suggesting that resistance is increased in sinusoidal capillaries. Shi *et al*[18] reported that HAP is higher and PVP is lower in rats with micro metastasis.

PERFUSION IMAGING OF HEPATIC HEMANGIOMA

Hemangioma is one of the common benign liver tumors; however, it is sometimes misdiagnosed as a malignant tumor.

Wang *et al*[19] used CTP to discriminate liver carcinoma and hemangioma and showed that the HAP in center and edge of liver carcinoma is 0.70 ± 0.23 mL/min per millilitre and 0.39 ± 0.22 mL/min per millilitre, respectively, which are significantly higher than those in normal liver. In addition, the HAP in center and edge of hemangioma is 0.14 ± 0.09 mL/min per millilitre and 0.50 ± 0.21 mL/min per millilitre, respectively, suggesting that comparison between the HAP in center and edge of liver carcinoma and hemangiom is important in discriminating liver carcinoma and hemangioma.

PERFUSION IMAGING IN LIVER TRANSPLANTATION

It is very important to evaluate non-invasive liver perfusion after liver transplantation. CTP can monitor the tendency of hemodynamic changes in portal vein and hepatic artery, contributing to the early diagnosis of blood vessel complications after transplantation.

Huang *et al*[20] detected perfusion parameters in 9 patients after liver transplantation for end stage liver disease, and found that the PVP in transplanted and control groups is 1.5763 ± 0.4540 mL/min per millilitre and 1.1885 ± 0.3899 mL/min per millilitre, respectively, while the TLP is 1.9594 ± 0.5727 mL/min per millilitre and 1.4712 ± 0.4451 mL/min per millilitre, respectively. The PVP and TLP in transplanted group was significantly increased (P = 0.044 and 0.036). No significant deviation was found in HAP and HPI. However, different data have also been reported[21]. HAP and HPI in transplanted and control group were 0.16 mL/min per millilitre, 0.25 mL/min per millilitre and 0.12, 0.16, respectively. Both parameters had a statistical significance. Huang *et al*[20] hold that it is related to the selection of patients, and examination time after transplantation, *etc*. Therefore, hemodynamic change after transplantation is a dynamic process, and different patients have different characteristics of hemodynamics.

APPLICATION OF CTP IN TREATMENT

CTP also plays an important role in therapeutic effect evaluation of liver diseases. Weidekamm *et al*[4] determined the perfusion parameters of liver parenchyma with dynamic single-section CT in patients with liver cirrhosis before and after transjugular intrahepatic portosystemic shunt (TIPS) and found that TIPS significantly increases HAP and TLP, but does not increase PVP.

Li *et al*[22] evaluated the changes in hepatic perfusion after interventional obliteration in patients with cirrhosis and portal hypertension using spiral CT perfusion imaging, demonstrating that partial spleen embolization (PSE) only increases HAP, while PSE in combination with percutaneous transhepatic obliteration (PTO) significantly increase HAP and TLP.

Tsushima *et al*[23] observed hemodynamic changes in hepatic parenchyma induced by transcatheter arterial embolization (TAE) for hepatocellular carcinoma in 22 patients. The HAP was increased 2-6 d after TAE (0.146 ± 0.073 mL/min per millilitre, P < 0.0002) compared with that before TAE (0.064 ± 0.039 mL/min per millilitre), but was decreased one month after TAE (0.086 ± 0.038 mL/min per millilitre). The PVP was decreased 2-6 d after TAE (0.541 ± 0.180 mL/min per millilitre, P < 0.001) compared with that before TAE (0.733 ± 0.263 mL/min per millilitre) and remained almost unchanged one month after TAE (0.651 ± 0.214 mL/min per millilitre),

suggesting that such perfusion changes are due to acute inflammatory responses.

Kan *et al*[24] quantified tumor perfusion in rats after TAE, and found that functional CT can detect changes in tumor perfusion after TAE, and MTT is increased because of obstruction of tumor vessels and reduced BF after TAE. TAE-associated hypoxia stimulates tumor angiogenesis and increases vascular permeability. Changes in permeability-surface area products are similar to those in BF, suggesting that measurement of tumor permeability after TAE is an important means for assessing the therapeutic effect and tumor angiogenic response that may help design a novel antivascular therapy that combines TAE and antiangiogenic therapy.

In summary, liver CT perfusion imaging has opened up a new area for its clinical application. One single CT scan can provide both morphologic and functional information, so that clinicians can detect the disease before morphological changes and evaluate the effect of treatment.

Multi-slice CT has a high time and spatial resolution for the measurement of perfusion. As an effective non-invasive method, CT perfusion imaging is safe, reproducible, easy to operate, etc. Whole liver perfusion will come true due to the development of multi-slice CT. We can get complete perfusion parameters of the entire liver. Therefore, CT perfusion imaging will be increasingly used in clinical practice.

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ORIGINAL ARTICLES

A rabbit model of pediatric nonalcoholic steatohepatitis: The role of adiponectin

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Abstract

AIM: To create a rabbit model of pediatric nonalcoholic steatohepatitis (NASH) and to evaluate the role of adiponectin in the process.

METHODS: Thirty-two specific pathogen-free male New Zealand rabbits were divided randomly into three groups: (1) the normal control group (n = 10) was fed with standard diet for 12 wk; (2) the model group A (n = 11); and (3) model group B (n = 11) were fed with a high-fat diet (standard diet + 10% lard + 2% cholesterol) for 8 and 12 wk, respectively. Hepatic histological changes were observed and biochemical parameters as well as serum levels of adiponectin, interleukin (IL)-6, IL-10 and tumor necrosis factor (TNF)-α were measured.

RESULTS: Typical histological hepatic lesions of NASH were observed in both model groups described as liver steatosis, liver inflammatory infiltration, cytologic ballooning, perisinusoidal fibrosis and overall fibrosis. Compared with the normal control group, there were significant increases in model groups A and B in weight gain (1097.2 ± 72.3, 1360.5 ± 107.6 *vs* 928.0 ± 58.1, P < 0.05, P < 0.01), liver weight (93.81 ± 6.64, 104.6 ± 4.42 *vs* 54.4 ± 1.71, P < 0.01), Lg (ALT) (1.9 ± 0.29, 1.84 ± 0.28 *vs* 1.60 ± 0.17, P < 0.01), and Lg (TG) (1.03 ± 0.24, 1.16 ± 0.33 *vs* 0.00 ± 0.16, P < 0.01). Weight gain was much more in model group B than in model group A (1360.5 ± 107.6 *vs* 1097.2 ± 72.3, P < 0.05). But, there was no significant difference between the two groups concerning the other indexes. Pro-inflammatory cytokines (IL-6 and TNF-α) increased in model group B compared with that of control and model group A (IL-6: 1.86 ± 0.21 *vs* 1.41 ± 0.33, 1.38 ± 0.42, P < 0.01; TNF-α: 1.18 ± 0.07 *vs* 0.66 ± 0.08, 0.86 ± 0.43, P < 0.01, P < 0.05), whereas serum adiponectin and IL-10 decreased in model groups compared with that in the control (adiponectin: A: 21.87 ± 4.84 and B: 21.48 ± 4.60 *vs* 27.36 ± 7.29, P < 0.05. IL-10: A: 1.72 ± 0.38 and B: 1.83 ± 0.39 *vs* 2.26 ± 0.24, P < 0.01). Lg (TC) and the degree of liver fatty infiltration was an independent determinant of serum adiponectin level analyzed by stepwise multiple regressions, resulting in 29.4% of variances.

CONCLUSION: This rabbit model produces the key features of pediatric NASH and may provide a realistic model for future studies. Adiponectin level partially reflects the severity of liver steatosis, but not the degree of liver inflammation.

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Key words: Nonalcoholic steatohepatitis; Pediatric animal model; Adiponectin; Interleukin 6; Tumor necrosis factor

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is recognized as the most common cause of liver disease in

pediatrics, paralleling the rapid rise in prevalence of obesity in children globally. Cases have been reported in obese children with liver steatosis and fibrosis by liver biopsy[1,2]. The study of the pathogenic or therapeutic factors involved in childhood nonalcoholic steatohepatitis (NASH) has been hampered by the absence of a suitable experimental model. The existing models were either using rats with a genetic defect[3], or lack of pathogenic factors such as cytochrome P4502E1 (CYP2E1) in which rats were required to be treated for a long time (up to 1 year)[4], and were fed with a diet lacking choline and methionine[5], creating a nutritional deficiency that is not common in patients with NASH. High fat diet-induced NASH may be a good model close to human conditions[6]. Rats have very short pre-pubertal stage and soon develop into adulthood in one month, therefore, it is not an ideal animal model to reflect the physiological and pathological state of children. However, the current theories about the pediatric NASH were all from the adult rats or from the clinical studies. Rabbits have at least 8-mo pre-pubertal stage and are supposed to be more ideal for mimicking pediatric NASH. Rabbit NASH animal model was only seen in Otogawas' research[7], but it did not investigate the pediatric NASH as its primary goal.

Adiponectin, one kind of adipocytokine, is known to modulate insulin effects. In the liver, it increases the sensitivity of insulin to inhibit gluconeogenesis and regulates hepatic nonesterified fatty acid (NEFA) metabolism *via* activation of NEFA oxidation and suppression of lipogenesis[8-10]. In our previous study and other clinical studies, serum adiponectin levels were inversely associated with body mass index (BMI) and NAFLD, and were positively associated with high-density lipoprotein (HDL)-cholesterol levels, which suggested that adiponectin might be a protective factor in NAFLD in obese children[11,12]. As in nonalcoholic steatohepatitis, endotoxins and certain endotoxin-inducible cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10, are also thought to play a role in the process of NASH[13]. In order to further understand the role of adiponectin in the process of childhood NASH and its relationship with the severity of the disease, we used a high-fat diet induced animal model to evaluate the role of serum adiponectin in the process of NASH, and to evaluate cytokines such as IL-6, IL-10 and TNF-α in the pathologic changes in NASH.

MATERIALS AND METHODS

Animal experiment

Thirty-two specific pathogen-free male New Zealand rabbits with a weight of 827 ± 36 g (mean ± SE) and 4-6 wk old, were obtained from the Experimental Animal Department of Zhejiang University School of Medicine (Hangzhou, China). The animals were divided randomly into three groups, each group matched in body weight and age: (1) the normal group (n = 10) was fed with standard diet for 12 wk; (2) the model group A (n = 11) and (3) model group B (n = 11) were fed a high-fat diet (standard diet + 10% lard + 2% cholesterol) for 8 and 12 wk, respectively. All rabbits were fed freely, given tap water, and kept in a room with controlled temperature (22 ± 1℃) and humidity (65%-70%). All animals received humane care and study protocols comply with the institutional guidelines. At the end of 8 and 12 wk, after one night fasting, rabbits were weighed and blood samples were collected through external jugular vein puncture after general anesthesia by injection of 1 mL/kg 3% sodium pentobarbital into the vein of ears. Rabbits were then sacrificed sequentially by an overdose of sodium pentobarbital injection. Intact livers were taken out of the abdominal cavity and washed with ice-cold 0.9% saline. The weight of livers was measured. Hepatic index was calculated (wet weight of liver/body weight). The right lobes of the livers were dissected out and pieces soaked in 10% neutral buffered formalin for histological observation by HE, red-oil and Masson trichrome staining. The diagnosis of NASH and classification of severity were made according to the criteria of Chinese Medical Association Committee of Nonalcoholic Fatty Liver Disease in the year of 2006[14].

Serum biochemical analysis

Serum transaminase (ALT and AST) activities, total cholesterol (TC), and triglyceride (TG) were determined on the Backman CX4 Automatic Biochemistry Analyzers using kits from Sigma. Serum adiponectin, IL-10, IL-6 and TNF-α were detected by commercially available ELISA kit (DiaClone, France).

Determinations of oxidative stress levels

The liver portions were homogenized in ice-cold 0.15 mol/L KCl. The degree of lipid peroxidation in the liver was assessed by measuring malondialdehyde (MDA) levels using the thiobarbituric acid (TBA) method, and superoxide dismutase (SOD) activities using the xanthine oxidase method according to the manufacturers' instructions. The assay kits for determining MDA and SOD were from Jiancheng Bioengineering Institute (Nanjing, China). Protein levels were determined by Coomassie brilliant blue, using bovine serum albumin as a standard.

Classification of the degree of liver inflammation and steatosis

Formalin-fixed and paraffin-embedded livers were processed routinely for hematoxylin and eosin, red-oil and Masson trichrome staining. Lobular inflammatory activity and severity of liver steatosis were determined according to the criteria of Chinese Medical Association Committee of Fatty Liver Disease in 2006 and Nouchi *et al*[15,16]. The inflammatory scores were leveled as Grade 1: focal collections of mononuclear inflammatory cells; Grade 2: diffuse infiltrates of mononuclear inflammatory cells; Grade 3: focal collections of polymorphonuclear cells in addition to mononuclear cell infiltrates; and

Grade 4: diffuse infiltrates of polymorphonuclear cells in the parenchymal area or lobular area. Severity of liver steatosis: Grade 1: < 30%; Grade 2: 30%-50%; Grade 3: 51%-75%; and Grade 4: > 75% under the low power microscope detection. Hepatocellular ballooning, Grade 0: absent; Grade 1: mild; Grade 2: marked. Perisinusoidal fibrosis, Grade 0: absent; Grade 1: up to 33%; Grade 2: 34%-66%; and Grade 3: > 66%. Overall fibrosis, Stage 1: none; Stage 2: either pericentral/pericellular or focal portal fibrosis; Stage 3: bridging fibrosis (central to central/central to portal); and stage 4: cirrhosis.

Statistical analysis

Statistical analyses were conducted using SPSS software (version 10.0). Quantitative data were presented as mean ± SD. Skewed distribution data were first logarithm transformed and tested for normality of distribution by examination of skewness and kurtosis. The statistical significance between means was estimated by one-way ANOVA followed by LSD multiple comparisons or an unpaired Student's t test where appropriate. Stepwise multiple linear regression models were used to examine the determinant of serum adiponectin. In the analysis of histological grading, nonparametric tests (Kruskal-Wallis *H* test) were used. A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Twenty-two male New Zealand rabbits were fed with a high-fat diet (standard diet + 10% lard + 2% cholesterol). All animals developed NASH at the end of 8 and 12 wk, according to the criteria of NASH diagnosis. Liver histology showed different grades of inflammation marked with different degree of mononuclear and polymorphonuclear cell infiltration, hepatocellular ballooning, and some showed portal and (or) perisinusoidal fibrosis.

Effects of fat diet on body weight, liver weight, weight gain and liver index

All rabbits completed the 8 wk or 12 wk experiment. There were no significant differences in the initial body weight among the three groups ($P > 0.05$). At the end of 8 wk or 12 wk, the liver weight and liver index (liver weight/body weight) were all significantly higher than that in control, but no difference was found between the two fat diet groups. Body weight gain (weight gain = body weight before death-initial body weight) increased stepwise in the control, group A (8 wk) and group B (12 wk) ($P < 0.05$) (Table 1).

Serum biochemical parameters

Serum transaminase (ALT, AST) and lipids (TG, TC) elevated remarkably, while serum adiponectin and IL-10 decreased significantly in two model groups, compared with the control. No difference was found between the two model groups. Pro-cytokines (IL-6 and TNFα) increased significantly in group B, compared either with group A or the control group ($P < 0.01$), but no difference was found between group A and control group. There was no difference in ALT/AST among all three groups (Table 1).

Table 1 Comparison of indexes among three groups

	Control (n = 10)	Group A (n = 11)	Group B (n = 11)
BW (g)	809.0 ± 58.0 (625-1067)	973.0 ± 86.39 (545-1350)	816.1 ± 67.5 (545-1200)
WG (g)	928.0 ± 58.1 (570-1268)	1097.2 ± 72.3[a] (779.0-1485)	1360.5 ± 107.6[c] (537-1795)
LW (g)	54.4 ± 1.71 (47-67.5)	93.81 ± 6.64[b] (62.2-121.9)	104.6 ± 4.42[b] (74.4-123.5)
Liver index	0.032 ± 0.001 (0.02-0.04)	0.045 ± 0.002[a] (0.03-0.06)	0.051 ± 0.005[a] (0.04-0.1)
Adiponectin (μg/L)	27.36 ± 7.29 (18.14-39.55)	21.87 ± 4.84[a] (12.12-28.47)	21.48 ± 4.60[a] (16.71-32.16)
ALT/AST	1.00 ± 0.62	0.73 ± 0.38	0.70 ± 0.1
Lg (ALT)	1.60 ± 0.17	1.9 ± 0.29[b]	1.84 ± 0.28[b]
Lg (AST)	1.67 ± 0.18	2.08 ± 0.31[b]	2.15 ± 0.30[b]
Lg (TC)	0.24 ± 0.16	1.56 ± 0.46[b]	1.56 ± 0.96[b]
Lg (TG)	0.00 ± 0.16	1.03 ± 0.24[b]	1.16 ± 0.33[b]
Lg (IL-10)	2.26 ± 0.24	1.72 ± 0.38[b]	1.83 ± 0.39[b]
Lg (IL-6)	1.41 ± 0.33	1.38 ± 0.42	1.86 ± 0.21[b,d]
Lg (INF-α)	0.66 ± 0.08	0.86 ± 0.43	1.18 ± 0.07[b,c]

Group A: 8 wk model; Group B: 12 wk model. Compared with control, [a]$P < 0.05$, [b]$P < 0.01$. Compared with group A, [c]$P < 0.05$, [d]$P < 0.01$. BW: Body weight; WG: Weight gain; LW: Liver weight.

Table 2 Hepatic malondialdehyde (MDA) levels and hepatic superoxide dismutase (SOD) activities among three groups

Group	MDA (nmol/mg protein)	SOD (U/mg protein)
Control	2.73 ± 0.45	36.39 ± 5.2
Group A	4.84 ± 0.72[b]	39.08 ± 3.5[b]
Group B	5.51 ± 0.91[b]	41.28 ± 3.2[b]

[b]$P < 0.01$ compared with control. There is no difference between group A and group B.

Determinations of oxidative stress levels and histological examinations

MDA levels and SOD activities were significantly increased in groups A and B, compared to the control group (Table 2). Histological examinations showed normal liver or mild microvesicular steatosis without inflammatory infiltration or fibrosis in the normal group. Mild to severe macro- and micro-vesicular steatosis, infiltration of mild lobular mixed neutrophilic and mononuclear cells, even focal necrosis, perisinusoidal and portal fibrosis, severe hepatocellular ballooning were observed in the 8 wk or 12 wk model groups ($P < 0.01$). The liver index, hepatic cell steatosis, inflammatory activity score and overall fibrosis stage were all significantly higher in the two model groups than in the control group. Liver steatosis and inflammatory infiltration were more severe in model group B than in model group A ($P < 0.05$), but no difference was found in other indexes between the two model groups ($P > 0.05$) (Table 3, Figures 1-2).

Table 3 Comparison of histopathology among three groups

	Group	0	1	2	3	4	χ^2
Steatosis	Control		10	0	0	0	
	Model A		0	1	3	7	17.6[b]
	Model B		0	1	1	9	18.4[b] 7.51[d]
Inflammatory score	Control		10	0	0	0	
	Model A		3	3	1	4	17.3[b]
	Model B		2	4	0	5	17.2[b] 4.9[c]
Cytologic ballooning	Control	9	1	0	0	0	
	Model A	0	5	6	0	0	10.8[b]
	Model B	0	3	8	0	0	11.1[b]
Perisinusoidal fibrosis	Control	10	0	0	0	0	
	Model A	0	8	3	0	0	17.9[b]
	Model B	0	7	4	0	0	17.6[b]
Overall fibrosis	Control		10	0	0	0	
	Model A		5	6	0	0	17.5[b]
	Model B		4	7	0	0	17.6[b]

Compared with control, [a]$P < 0.05$, [b]$P < 0.01$. Compared with model A, [c]$P < 0.05$, [d]$P < 0.01$.

Table 4 Pearson correlation of adiponectin with other variants

	Liver steatosis	Lg (TC)	Lg (Tg)	Lg (TNF-α)	Liver index
Adiponectin	-0.303	-0.424	-0.33	-0.412	-0.313
P value	0.046	0.008	0.03	0.01	0.04

Table 5 Degree of liver cell steatosis and Lg (TC) by stepwise multiple regression analysis

	B	*t*	*P*	R^2
Constant	27.890	14.322	0.000	
Lg (TC)	-1.333	2.978	0.006	
Liver steatosis	-0.97	2.167	0.038	0.294

Association between serum adiponectin and other factors

Serum adiponectin levels were inversely associated with liver steatosis (r = -0.303, P = 0.046), Lg (TC) (r = -0.424, P = 0.008), Lg (TG) (r = -0.33, P = 0.03), Ig (TNF-α) (r = -0.412, P = 0.01) and liver index (r = -0.313, P = 0.04) (Table 3), but not related to the degree of liver inflammatory infiltration, wet liver weight, Lg (ALT), Lg (AST), Lg (IL-10) and Lg (IL-6). The degree of liver cell steatosis and Lg (TC) were independent determinants of serum adiponectin levels analyzed by stepwise multiple regression analysis, which resulted in a 29.4% variance (Tables 4 and 5).

DISCUSSION

By feeding rabbits a high-fat diet, we produced the typical hepatic lesions of NASH. The distinctive morphological histological features of NASH included steatosis and lobular inflammation, which contained

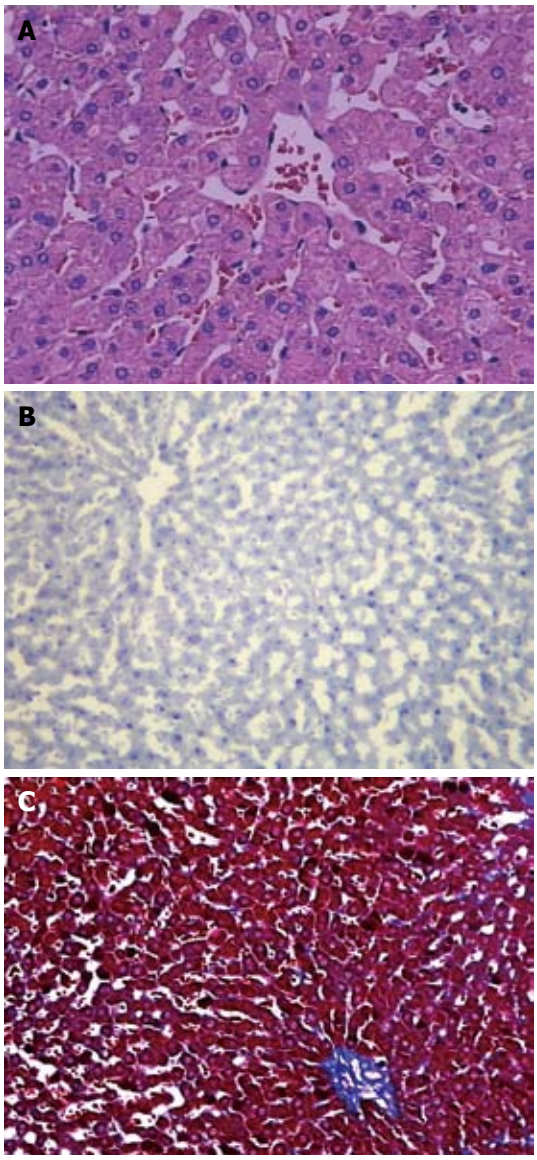

Figure 1 The rabbits fed with the standard diet. A: Normal liver cells without vacuolar degeneration and no distinct inflammatory cell infiltration by HE staining (× 400); B: No positive fat deposition by red-oil staining (× 100); C: Normal liver cells without apparent collagen fibrosis by Masson trichrome staining (× 100).

polymorphonuclear leukocytes and perisinusoidal fibrosis in zone 3 of the acinus. Other common features were also found such as hepatocellular ballooning and poorly formed Mallory's hyaline. Moreover, the MDA levels and SOD activities were significantly increased in the model groups demonstrating oxidative stress in the pathogenesis of NASH. Together with elevation of serum transaminases and lipids, we successfully established an animal model of pediatric NASH. Model A is not significantly different from model B in serum biochemical analysis and main histological changes.

Adiponectin is much implicated in the pathogenesis of NAFLD/NASH. We found that serum adiponectin was much lower in the model groups as the pathologic changes showed much deteriorated stetosis, inflammatory infiltration and overall fibrosis in the latter. It was in agreement with our previous study and other studies that

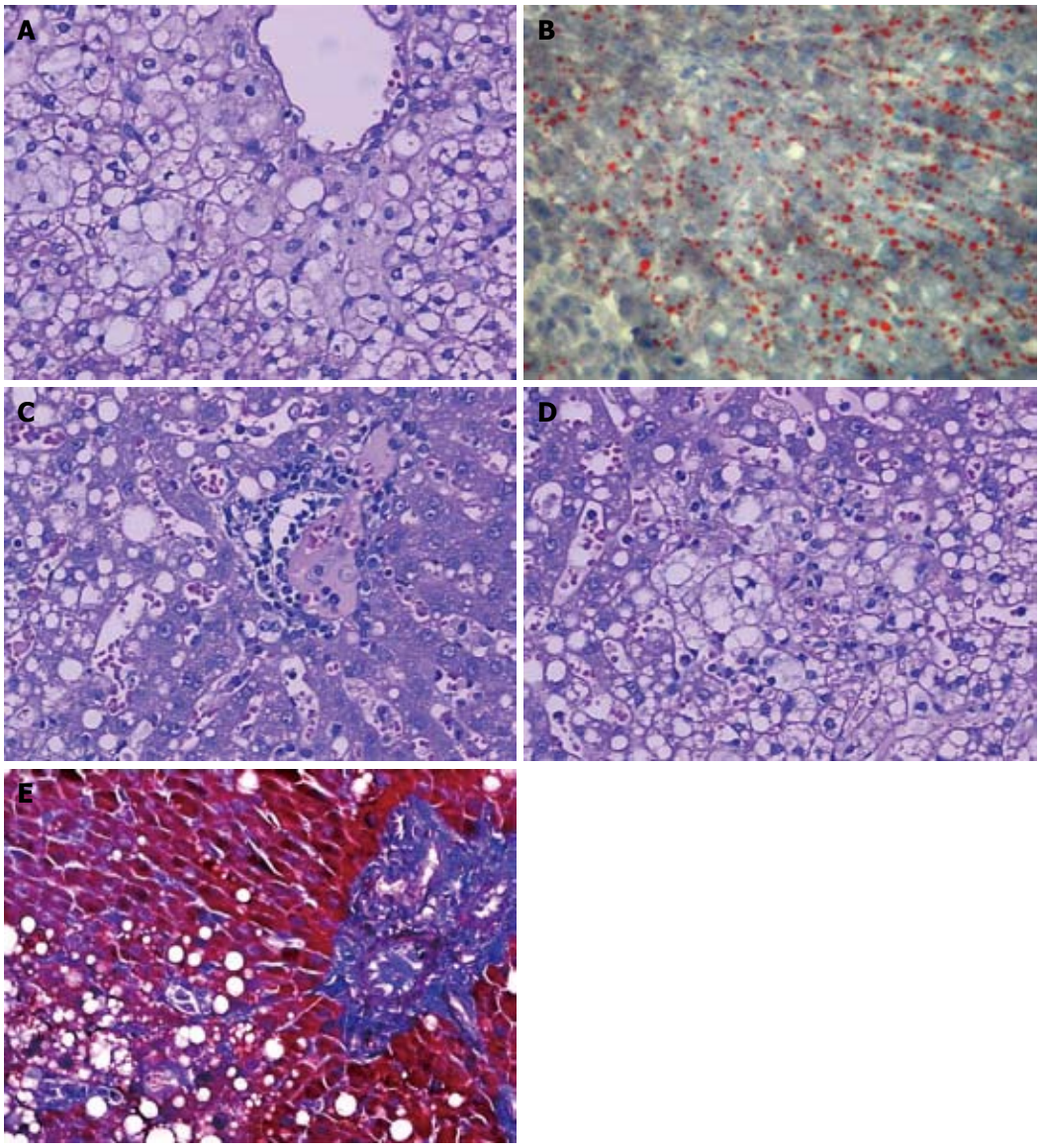

Figure 2 The rabbits fed with the high-fat diet. A: Pronounced hepatic steatosis. Hepatocellular ballooning with clear vacuolar degeneration was apparent by HE staining (× 400); B: Pronounced hepatic steatosis with positive fat infiltration by red-oil staining (× 200); C: Abundant mononuclear and polymorphonuclear inflammatory cells infiltrates by HE staining (× 400); D: Pieces necrosis by HE staining (× 400); E: Significant collagen and reticulin fibrosis among hepatic cells in blue color by Masson trichrome staining (× 200).

serum adiponectin values were lower in NASH patients than in non-NASH persons, and hepatic expression of adiponectin and its type Ⅱ receptor were also less in NASH than those in fatty liver[17-19]. Furthermore, reduced adiponectin levels were associated with increased fat content and extensive necroinflammation in NAFLD patients[20]. In our models, adiponectin was inversely associated with liver steatosis, Lg (TC), Lg (TG) and liver index, but not related to the degree of liver inflammatory infiltration. Stepwise multiple regression analysis showed that the degree of liver cell steatosis and Lg (TC) was independent determinants of serum adiponectin levels, which resulted in a 29.4% variance. These coincide with other studies. Available evidence from cross-sectional studies suggests that adiponectin levels in human are strictly associated with the amount of centrally located fat[21,22]. And clinical imaging studies also support a physiological link between adiponectin and liver fat accumulation[23,24]. Hypoadiponemia may predict a high percentage of hepatic fat content, but not the score of necroinflammation or fibrosis. The actions of adiponectin on the liver are supposed to oppose fatty acid synthesis and promote mitochondrial β-oxidation, which are thought to exert through activation of the cyclic-AMP dependent protein kinase (AMPK)[25]. Whether a single fasting adiponectin value can reliably distinguish an individual patient with NASH from someone with only steatosis or early fibrosis needs to be evaluated in the future studies.

Adiponectin has anti-inflammatory properties in the liver, and its deficiency might account for high aminotransferase and liver disease progression. However, we could not confirm a direct association between adiponectin and ALT. Our study does not support the findings of a recent pediatric study which demonstrated a correlation between ALT and adiponectin in obese subjects with or without abnormal ALT[26]. This suggests that although adiponectin has an anti-inflammatory effect, it cannot predict the inflammatory severity and deterioration of aminotransferase in NASH. In NASH, oxidative stress causes various types of functional and structural damage and frequently increases proinflammatory cytokine production. Serum levels of TNF-α and IL-6 increased stepwise in the control and

model groups, while adiponectin and IL-10 markedly decreased in the two model groups. This is in agreement with the studies that cytokines producing cells in ob/ob livers are Th1 polarized, while Th2 anti-inflammatory cytokines were down-regulated[27]. This may be also due to the loss of the protective effect of adiponectin since it was found to have anti-inflammatory effects by opposing the synthesis and release of TNF-α from macrophages within adipose tissue in obesity[28]. Adiponectin may also have the effect of inducing the regulatory T cells in its tolerate state by mainly producing the Th2 cytokine of IL-10. Thus the imbalance of protective and proinflammatory cytokines probably contributes to the histological inflammation and steatosis in NASH. Moreover, some studies have shown that administration of exogenous adiponectin reverses experimental forms of NAFLD and steatohepatitis[29]. However, the cross talk among the immune cells (especially T cells), cytokines and endocrine cells need to be clarified in the future studies.

In conclusion, the pediatric rabbit NASH model has been successfully produced in this study, which may provide a realistic model for future studies. Adiponectin level partially reflects the severity of liver steatosis, but not the degree of liver inflammation. The imbalance of anti-inflammatory cytokines (adiponectin, IL-10) and proinflammatory cytokines (TNF-α, IL-6) may contribute to the histological process in NASH.

COMMENTS

Background

The study of the pathogenic or therapeutic factors involved in childhood nonalcoholic steatohepatitis (NASH) has been hampered by the absence of a suitable experimental model. The existing models were either using rats with a genetic defect, or lack of pathogenic factors such as cytochrome P4502E1 (CYP2E1), or involved feeding rats a diet lacking choline and methionine, which all were not the natural course of developing NASH in patients. High fat diet-induced NASH may be a good model close to human conditions. Rabbits have at least an 8-mo pre-pubertal stage and are supposed to be more ideal for mimicking peadiatric NASH. Adiponectin, one kind of adipocytokine, was known to modulate insulin effects. But its role in the process of peadiatric NASH and its relationship with the severity of the disease is not clear.

Research frontiers

Nonalcoholic fatty liver disease is now recognized as the most common cause of liver disease in pediatrics, paralleling the rapid rise in prevalence of obesity in children globally. Most studies focus on the mechanisms of NASH such as "second hit theories", the pathogenic changes in NASH, or efforts on the therapeutic areas. However it is still a "bottle neck" to establish a suitable animal model.

Innovations and breakthroughs

For the first time, an easier and less expensive high-fat diet induced pediatric NASH model has been successfully produced in this study. Moreover, the study of the cytokine adiponectin in the process of the disease showed that it partially reflects the severity of liver steatosis but not the degree of liver inflammation. The imbalance of anti-inflammatory cytokines (adiponectin, IL-10) and proinflammatory cytokines (TNF-α, IL-6) may contribute to the histological process in NASH.

Applications

This high-fat diet induced rabbit NASH model may provide a realistic pediatric NASH model for future studies.

Peer review

Development of a simple and inexpensive rodent model of NASH is an important issue in the study of the etiology of this disease. As a model of NASH, the rabbit model looks promising and this manuscript may represent an important contribution to this field. Another major contribution is the development of fibrosis in a simple model with a high fat diet for 8 wk.

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Aspirin increases susceptibility of *Helicobacter pylori* to metronidazole by augmenting endocellular concentrations of antimicrobials

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Abstract

AIM: To investigate the mechanisms of aspirin increasing the susceptibility of *Helicobacter pylori* (*H pylori*) to metronidazole.

METHODS: *H pylori* reference strain 26695 and two metronidazole-resistant isolates of *H pylori* were included in this study. Strains were incubated in Brucella broth with or without aspirin (1 mmol/L). The *rdxA* gene of *H pylori* was amplified by PCR and sequenced. The permeability of *H pylori* to antimicrobials was determined by analyzing the endocellular radioactivity of the cells after incubated with [7-^{3}H]-tetracycline. The outer membrane proteins (OMPs) of *H pylori* 26695 were depurated and analyzed by SDS-PAGE. The expression of 5 porins (hopA, hopB, hopC, hopD and hopE) and the putative RND efflux system (hefABC) of *H pylori* were analyzed using real-time quantitative PCR.

RESULTS: The mutations in *rdxA* gene did not change in metronidazole resistant isolates treated with aspirin. The radioactivity of *H pylori* increased when treated with aspirin, indicating that aspirin improved the permeability of the outer membrane of *H pylori*. However, the expression of two OMP bands between 55 kDa and 72 kDa altered in the presence of aspirin. The expression of the mRNA of hopA, hopB, hopC, hopD, hopE and hefA, hefB, hefC of *H pylori* did not change when treated with aspirin.

CONCLUSION: Although aspirin increases the susceptibility of *H pylori* to metronidazole, it has no effect on the mutations of *rdxA* gene of *H pylori*. Aspirin increases endocellular concentrations of antimicrobials probably by altering the OMP expression.

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Key words: *Helicobacter pylori*; Aspirin; Metronidazole; Resistance; Minimum inhibitory concentrations

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INTRODUCTION

Aspirin, referred to as non-steroidal anti-inflammatory drugs (NSAIDs), is one of the most widely used drugs worldwide. It inhibits cyclooxygenases (COX), thereby irreversibly blocking the conversion of arachidonic acid to prostanoids. In addition, aspirin is also considered to offer some protection against coronary heart disease[1], due in part to inhibition of the thromboxane A2, a potent platelet aggregator. It has been reported that aspirin demonstrates chemopreventative activity against cancers in the esophagus, stomach and colon by inducing apoptosis in epithelial cells and regulating angiogenesis[2-4]. Aspirin also has numerous effects in different bacterial species. Previous studies reported that aspirin could inhibit the growth of some bacteria, affect the production of virulence factors of some bacteria,

and alter the susceptibility of bacteria to some antibiotics by influencing the gene expression and inducing a number of morphological and physiological alterations in bacteria[5].

We previously reported that NSAIDs, including sodium salicylate, aspirin, indomethacin and celecoxib, inhibited the growth of *H pylori* in a dose-dependent manner when incubated in brucella broth *in vitro*[6-9]. These drugs also significantly affected the activity of virulence factors of *H pylori*, for example, urease and vaculating cytotoxin[8,9]. In addition, the minimum inhibitory concentrations (MICs) of clarithromycin, metronidazole and amoxicillin to *H pylori* decreased when treated with a low concentration of aspirin[6-8], indicating that aspirin increased the susceptibility of *H pylori* to these antimicrobials.

The aim of the present study was to investigate the mechanisms of aspirin increasing the susceptibility of *H pylori* to metronidazole. The *rdxA* gene of *H pylori* treated with and without aspirin was analyzed by PCR amplification and sequencing. The effect of aspirin on the permeability of the outer membrane of *H pylori* was determined using [7-^{3}H]-tetracycline. The effects of aspirin on the expression of outer membrane proteins (OMPs) of *H pylori* were also determined.

MATERIALS AND METHODS

Chemicals

Aspirin (Sigma Chemical Co, St Louis, MO, USA) and proton conductor carbonyl cyanide m-chlorophenylhydrazone (CCCP, Sigma Chemical Co.) were dissolved in DMSO (Sigma Chemical Co.) in advance. [7-^{3}H]-tetracycline (0.6 Ci/mmol; 22.2 GBq/mmol; Dupont/NEN Research Products, Boston, Mass.) was freshly dissolved in thin hydrochloric acid.

Strains and culture conditions

H pylori reference strain 26 695 (susceptible to metronidazole) and two clinical isolates of *H pylori* (metronidazole resistant, R1 and R2) were included in this study. Strains were cultured on Columbia agar plates containing 8% sheep blood in a microaerobic atmosphere (10% CO_2 and 5% O_2) at 37℃ for 2-3 d. *H pylori* of 10^8 CFU/mL were then inoculated in 20 mL Brucella broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA) in a set of 10 cm Petri dishes with 1 mmol/L aspirin or with vehicle control (DMSO 0.1%). Dishes were placed in an anaerobic jar (Oxoid) and incubated at 37℃ on a shaker at 60 r/min under microaerobic conditions for 48 h.

Determination of MICs

Bacteria were prepared in Brain Heart Infusion broth to yield a viable count of 3×10^8 CFU/mL (equivalent to 1 McFarland turbidity standard unit) and used as the inocula for susceptibility testing. Bacterial suspension (100 μL) was spread, in duplicate, on Columbia agar plates with or without aspirin (1 mmol/L). A single E-test strip of metronidazole (OXOID Ltd, England) was applied to each plate. The MIC of metronidazole and the possible effect of aspirin on the MIC of metronidazole were determined after 72 h of incubation at 37℃ under microaerobic conditions.

Extraction of genomic DNA

H pylori genomic DNA was extracted using silicon dioxicide method. Cells were harvested and washed twice in phosphate-buffered saline (PBS) (0.01 mol/L, pH 7.2). The cell precipitation was suspended in 100 μL TE. Then 5 μL SiO_2 Liq. and 400 μL binding buffer (containing 4 mol/L guanidinium isothiocyanate, 50 mmol/L Tris-HCl, 20 mmol/L EDTA) was added and incubated at 55℃ for 5 min with shaking once every minute. The suspension was centrifuged at 8000 r/min for 30 s at room temperature and the precipitate was washed thrice in cleaning buffer (containing 20 mmol/L Tris-HCl, 1 mmol/L EDTA, 100 mmol/L NaCl and dehydrated alcohol). The resulting suspension was dried at 55℃ and stored at -20℃.

Amplification of H pylori rdxA gene and sequencing

The fragments (886 bp) containing the complete *rdxA* gene was amplified by PCR. Forward primer: 5'-AGG GATTTTATTGTATGCTACAA-3'; Reverse primer: 5'-AGGAGCATCAGATAGTTCTGA-3'. The PCR amplification was carried out in 25 μL reaction solution containing 2 μL of *H pylori* genomic DNA, 4.5 μL of 10 × PCR buffer (with 15 μmol/L $MgCl_2$), 2 μL of dNTPs (each 2.5 mmol/L), 2 μL of forward and reverse primers (each 5 μmol/L), 0.5 μL of TaqDNA polymerase (1 U/μL) and 9 μL of ddH_2O. The reaction was denatured initially at 94℃ for 5 min, followed by 30 cycles, with each cycle composed of 30 s at 94℃ (denaturation), 1 min at 52℃ (annealing), and 1 min at 72℃ (extension). After a final extension of 10 min at 72℃, the amplicons were electrophoresed in a 1.5% agarose gel and purified using the silicon dioxicide method as described above. The resulting *rdxA* gene was sequenced by the dideoxy chain termination procedure at Beijing Li-Jia-Tai-Cheng Technology Company. The *rdxA* genes of *H pylori* treated with and without aspirin were analyzed on line (http://align.genome.jp).

Uptake studies using [7-^{3}H] tetracycline

Strain 26 695 was grown to mid-logarithmic phase (approximately from 3×10^9 to 5×10^9 CFU/mL) in Brucella broth and then 1 mmol/L of aspirin or DMSO (< 1%, vehicle control) were added for 6 h at 37℃ on a shaker at 60 r/min under microaerobic condition. Cell suspension was centrifuged at 8000 r/min for 10 min at room temperature and the precipitate washed and suspended in HEPES buffer (pH 7.2, containing 100 μmol/L $MgCl_2$). At room temperature, 5 μCi [7-^{3}H]-tetracycline (0.6 Ci/mmol; 22.2 GBq/mmol; Dupont/NEN Research Products, Boston, USA) was added to 10 mL cell suspension. After 20 min, each cell suspension was divided into two halves, and 100 μmol/L CCCP was added to one half. One milliliter aliquots were

taken at 10 min intervals and washed three times in PBS. The resulting pellets were then diluted scintillation fluid and analyzed for radioactivity in an scintillation counter (TRI-CARB 2100TR).

Purification of OMPs

H pylori 26 695 was incubated in Brucella broth for 48 h. The suspension was centrifuged at 8000 r/min for 10 min, washed, and suspended in ice-cold Tris-Mg buffer (10 mmol/L Tris-HCl containing 5 mmol/L $MgCl_2$, pH 7.3) and sonicated (once 30 s at 3-5 s interval for 5 min) until most of the cells were disrupted as visualized microscopically. Unbroken cells were removed by centrifugation at 8000 r/min for 20 min at 4℃. The inner and outer membranes were concentrated by centrifugation at 50 000 r/min for 60 min at 4℃. The precipitate was suspended in 2% Triton Tris-Mg (pH 7.5) and incubated for 30 min at room temperature, and then centrifuged and incubated again under the same condition. The resulting pellets, OMPs, were washed twice in 10 mmol/L Tris-HCl and resolved in ddH_2O. The final concentration of OMPs was determined by Coomassie brilliant blue R250 method.

SDS-PAGE gel electrophoresis

Ten microgram OMPs were used for SDS-PAGE gel electrophoresis at permanent voltage (5% stacking gel at 60 V, 10% separating gel at 100 V). After incubation for 30 min in fixing liquid, the gel was dyed with Coomassie brilliant blue G250 for 30 min.

Isolation of total RNA and reverse transcription

Total RNA was obtained by the TRIzol method as described by manufacturer (Invitrogen, Burlington, Ontario, Canada), and the contaminating DNA was removed by DNase I treatment according to the manufacturer (Sigma). For cDNA synthesis, 4 μg RNA diluted with DEPC H_2O was heated to 70℃ for 5 min and chilled quickly on ice for 15 min. The samples were then added to a 20 μL reaction mixture containing 2 μL random hexameric primers (1 μg/μL), 0.4 μL of RNasin, 1 μL of M-MLV, 4 μL of dNTPs (each 2.5 mmol/L) and 4 μL of 5 × RT buffer. The cDNA synthesis reaction was performed for 60 min at 37℃. The enzyme was subsequently inactivated at 95℃ for 5 min. Aliquots of cDNA were stored at -70℃.

Real-time quantitative PCR

The mRNA levels of hopA, hopB, hopC, hopD, hopE and hefA, hefB, hefC were determined by real-time PCR using an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA.). Specific primers and TaqMan probes were designed with the aid of the Primer Express program 3.0 (Perkin-Elmer Applied Biosystems) (Table 1). A standard curve was constructed using 10-fold serial dilutions of each cDNA. Reaction mixtures for PCR (50 μL) were prepared by mixing 5 μL synthesized cDNA solution with 5 μL of 10 × PCR buffer (containing 15 μmol/L $MgCl_2$), 3 μL forward and reverse primers (each 5 μmol/L), 4 μL dNTPs (each 2.5 mmol/L), 2 μL TagMan probe, 1 μL of ROX, 0.5 μL TaqDNA polymerase (1 U/μL). PCR was carried out at 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s and 60℃ for 60 s according to the manufacturers' instructions. The levels of the protein mRNA were expressed as the ratio of the protein mRNA to 16S rRNA mRNA [protein mRNA (U/mL)/ 16S rRNA mRNA (U/mL) ratio × 100 000]. The PCR was carried out in quintuple using samples prepared at the same time.

Table 1 Primers and probes used in real-time quantitative PCR

Probe or primer type	Sequence (5'-3')
hopA F	ATCATGCTAGTGATGGCGTTAAAG
hopA R	CAGGCATAGACGGAGGCAAT
hopA Probe	CCAAAAATATTGCATGCGTTCCCGC
hopB F	CTTGGTGCAAAACATCGTCAAT
hopB R	CCCGCCATAGCTCACTTGAT
hopB Probe	TAACGCTAGCCAACAACGCTAACATCAGCA
hopC F	CGCTCTTTATAACGCGCAAGTAA
hopC R	GCTGTTCCCGGCTCTGAAT
hopC Probe	TGGATAAAATCAACGCGCTCAACAATCAG
hopD F	CTGCTTGAGCGCGGTTTAA
hopD R	CAACCTAGACACTGGGAAAGCAT
hopD Probe	CTTGCGCTCTAGCGTTAGCGAACATGC
hopE F	GGATTGCACAGGGAGTGTTGT
hopE R	GCCCCATTAGCGTATTTAGCAT
hopE Probe	TTGCCCCCCAGGTCTTACCGCT
hefA F	AGGGCGTTTTGGGAATTTCT
hefA R	GCATGATGGATTGTTTTTGCA
hefA Probe	CCCCGGTCAGCAAAATACGGCTG
hefB F	AGGGCGATGTTTTGTTGCTT
hefB I	CCCCCAATTTTGCTGTATCGT
hefB Probe	AATCAAGACAAACAGGCTCAAAGCGATTCC
hefC F	GTTTGCGTTCTTGCGTAACG
hefC R	TGTTTAATGAAAAGCCCATCCA
hefC Probe	CACGATCACCCTCGTTTCAGCGATC
16S rRNA F	CCGCCTACGCGCTCTTTAC
16S rRNA R	CTAACGAATAAGCACCGGCTAAC
16S rRNA Probe	CCCAGTGATTCCGAGTAACGCTTGCA

Statistical analysis

Statistical analysis was performed using SPSS, 13.0. Representative data of endocellular radioactivity and quantitative PCR were presented as mean ± SD. The Student's *t* test was used to compare data. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of aspirin on MICs of metronidazole

For strain R1, MIC of metronidazole decreased from 256 μg/mL to 0.25 μg/mL in the presence of aspirin (1 mmol/L), and for strain R2, MIC of metronidazole reduced from 64 μg/mL to below the readable value (0.016 μg/mL), indicating that aspirin increased the susceptibility of *H pylori* to metronidazole and converted these two resistant strains to susceptible strains.

Effects of aspirin on mutations of rdxA gene

The 886 bp DNA fragments containing the complete *rdxA* gene were amplified by PCR for *H pylori* reference

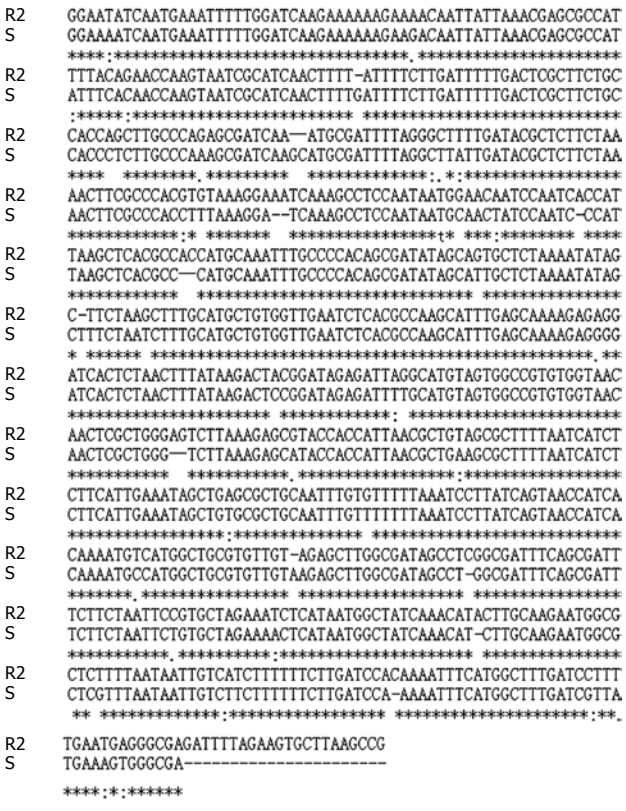

Figure 1 Sequence comparison on line (http://align.genome.jp) of the *rdxA* gene between metronidazole resistant strain (R2) and *H pylori* reference strain 26 695 (S).

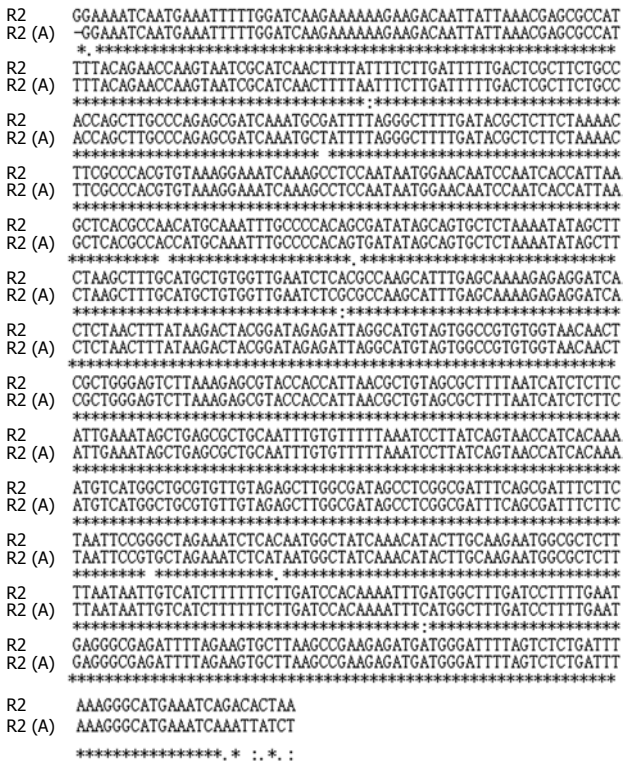

Figure 2 Sequence comparison on line (http://align.genome.jp) of the *rdxA* gene between metronidazole resistant strain (R2) and its corresponding susceptible strain in the presence of aspirin [R2 (A)].

strain 26 695 and the two metronidazloe resistant strains (R1, R2) treated with or without aspirin. Sequence analysis revealed that there were point mutations, insertions and deletions in *rdxA* gene of strain R2 when compared with the reference strain 26 695, and the nucleotide homology between the two strains (26 695 and R2) was 94.4%. However, the nucleotide homology of *rdxA* gene between strain R2 and its corresponding isolate R2(A) (strain R2 treated with aspirin) was over 99.5% (Figures 1 and 2).

Effects of aspirin on permeability of outer membrane of H pylori

Irrespective of the presence of CCCP, the radioactivity of *H pylori* treated with aspirin was higher when compared to that of the vehicle control (DMSO), indicating that aspirin enhanced the permeability of the outer membrane of *H pylori* to antimicrobial (Figure 3). Irrespective of the presence of aspirin, the radioactivity of *H pylori* cells decreased when the efflux pump inhibitor CCCP was added, indicating that aspirin did not interfere with the collapsion effect of CCCP on the proton gradient across the cytoplasmic membrane of *H pylori* (Figure 4).

Effects of aspirin on OMP profiles

The OMP profiles of *H pylori* treated with aspirin were similar to that in control. However, the expression of two OMPs (Band 1 and 2) between 55 kDa and 72 kDa altered in the presence of aspirin (Figure 5).

Table 2 cDNAs levels of porins and efflux system of *H pylori* tested by real-time quantitative PCR

Gene	Levels of cDNA		
	Aspirin group	DMSO control	Blank control
hopA	963.60 ± 23.33	1087.75 ± 26.42	856.27 ± 25.43
hopB	84.00 ± 15.97	91.45 ± 14.83	100.15 ± 16.28
hopC	52.98 ± 11.67	51.77 ± 12.01	52.79 ± 10.27
hopD	83.86 ± 14.09	92.46 ± 17.78	89.92 ± 13.72
hopE	54.07 ± 10.58	52.77 ± 11.93	52.12 ± 9.92
hefA	12.31 ± 2.12	12.51 ± 1.99	12.97 ± 2.76
hefB	25.47 ± 4.73	28.48 ± 5.07	25.60 ± 4.99
hefC	55.62 ± 8.89	52.08 ± 9.78	53.37 ± 10.04

$P > 0.05$ (Aspirin group compared with DMSO control or blank control).

Effects of aspirin on expression of mRNA of porins and efflux system

Real-time quantitative PCR indicated that there was no significant difference in the levels of the cDNA of hopA, hopB, hopC, hopD, hopE and hefA, hefB, hefC for strains treated with aspirin when compared with that in control. This indicated that, irrespective of the presence of aspirin, the expression of the levels of mRNA of hopA, hopB, hopC, hopD, hopE and hefA, hefB, hefC did not change (Table 2).

DISCUSSION

H pylori is a spiral-shaped gram-negative bacterium and an important human pathogen that colonizes in the stomach of 50% of the world human population[10,11].

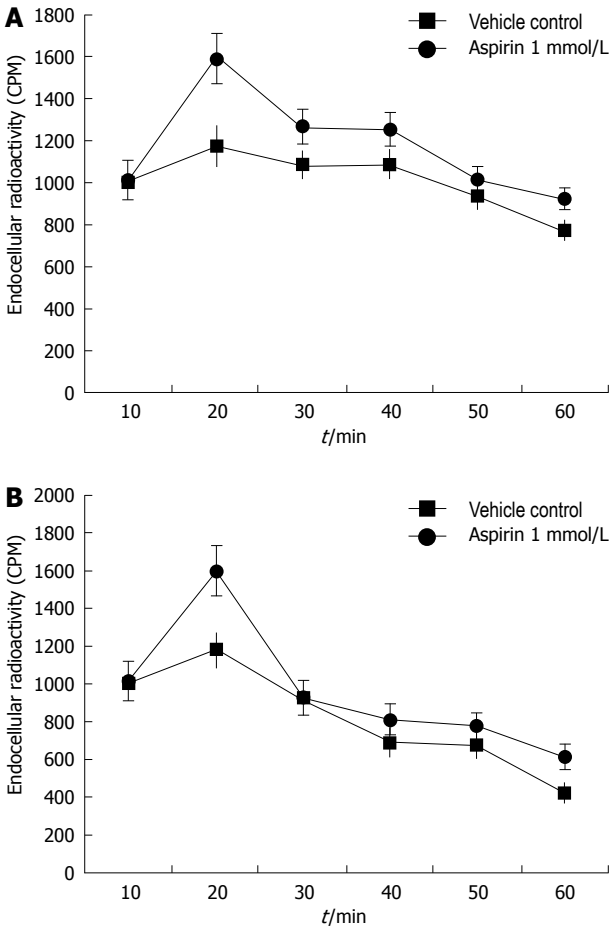

Figure 3 Radioactivity of *H pylori* cells treated with aspirin (1 mmol/L) or vehicle control (DMSO). A: CCCP; B: No CCCP.

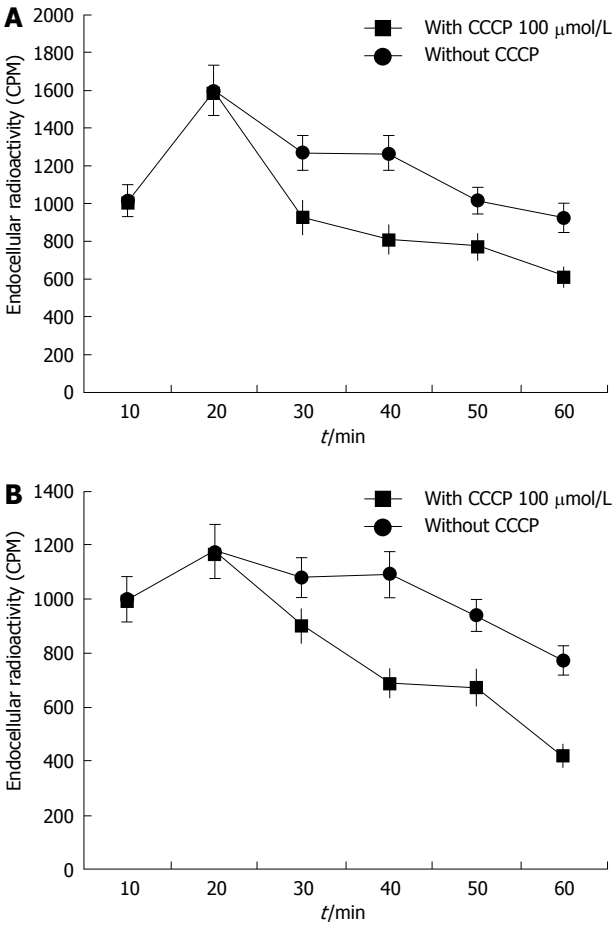

Figure 4 Radioactivity of *H pylori* cells treated with CCCP (100 μmol/L) or without CCCP. A: Aspirin; B: Vehicle control.

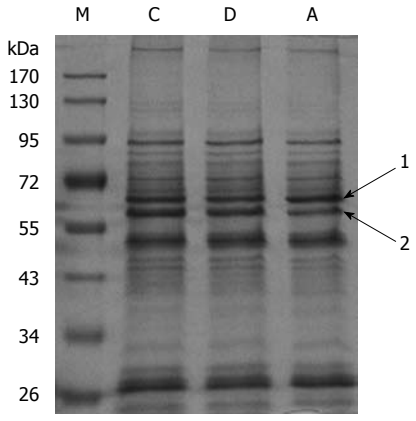

Figure 5 OMP profiles of *H pylori* 26695. M: Size marker; C: Control; D: DMSO; A: Aspirin. Band 1: OMP increased in the presence of aspirin; Band 2: OMP decreased in the presence of aspirin.

Infection results in chronic inflammation of the gastric mucosa and peptic ulcer, and it has been proven that *H pylori* infection is strongly associated with adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. International Agency for Research on Cancer affiliated to World Health Organization (WHO) defined *H pylori* as one of the first class human carcinogens[12]. Recent Studies revealed that *H pylori* infection plays important roles in the invasion of heart and brain vascular disorders, autoimmune diseases, nutritional and metabolic diseases, hematopathy and dermatologic diseases. Eradication of *H pylori* infection is very important to prevent and cure these diseases. The most successful treatment regimens use combinations of two or more antibiotics, such as amoxicillin, clarithromycin, metronidazole, or tetracycline, along with a proton pump inhibitor or bismuth. However, with the wide use of antimicrobials in clinical practice, antibiotic resistance is more and more apparent and is considered one of the major causes of treatment failure[13].

Early studies suggested that salicylate inhibited the growth of some bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*, and affected the activity of fimbriae, flagellum and the production of biofilm, slime, and thus might alter the pathogenicity of bacteria[14-21]. It has been reported that *in vitro*, salicylate could alter the susceptibilities of bacteria to some antimicrobials. Salicylate induced the intrinsic multiple antimicrobial resistance phenotype in many bacteria, such as *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus*, and increased the susceptibilities of some bacteria to aminoglycosides[22-29]. Our previous studies also found that *in vitro* aspirin not only inhibited the growth of *H pylori*[6-9], but also decreased the MICs of metronidazole, clarithromycin and amoxicillin to *H pylori*, and even converted some resistant strains to susceptible ones[6-8]. Therefore, the present study investigated the mechanisms of aspirin increasing the susceptibility of

H pylori to metronidazole.

At least four distinctive mechanisms of antibiotic resistance have been described in bacteria: enzymatic inactivation, decreased permeability of bacterial membranes, active efflux of antimicrobial agents, and alteration of target sites of antimicrobials to bacteria[30]. Metronidazole, clarithromycin and amoxicillin are different kinds of antimicrobials, and each has its different effect on different target site in *H pylori*. Likewise, resistance of *H pylori* to these antimicrobial agents arises through various mechanisms. A reasonable explanation of aspirin increasing the susceptibility of *H pylori* to antimicrobials with different antibacterial mechanisms is that aspirin does not alter the target sites of bacteria, but increases the endocellular concentrations of antimicrobials.

Metronidazole is a prodrug activated by nitroreductases in bacteria cells. Resistance of metronidazole is caused by either the absence or the inactivation of the nitroreductases[31]. It has been reported that the resistance of *H pylori* to metronidazole was mainly due to null mutations in the *rdxA* gene, which encoded an oxygen-insensitive NADPH nitroreductase[32]. However, studies also reported involvement of other reductases in the development of the resistant phenotype. In addition to oxygen-insensitive NADPH nitroreductases, several other nitroreductases in *H pylori*, such as NADPH flavin oxidoreductase, ferredoxin-like protein, flavodoxin, α-ketoglutarate oxidoreductase and pyruvate: flavodoxin oxidoreductase, have been found to reduce metronidazole and to generate active compounds[33,34]. In our study, mutations in *rdxA* gene might be involved for the resistance of the isolated strain (R2). However, in the presence of aspirin, the strain converted from metronidazole resistant to susceptible, while the mutations in *rdxA* gene did not change. By using isotope scintillation technique with [7-^{3}H]-tetracycline, our study revealed that aspirin increased the endocellular concentration of antimicrobials in *H pylori* cells, indicating that aspirin increased the outer membrane permeability of *H pylori* to antimicrobials. With the higher endocellular concentration in the presence of aspirin, metronidazole might be reduced and activated by other nitroreductases in *H pylori*. Therefore, the MIC of *H pylori* to metronidazole decreased, and in some circumstances, resistant strains even converted to susceptible ones.

Two pathways may be involved in the mechanisms for the increasing concentration of antimicrobials in bacteria cells. One is the augmentation of anti-microbials entering into the bacteria cells passively; the other is the impairment of antimicrobials pumping out of the bacteria actively. Previous studies on *Escherichia coli* revealed that salicylate increased resistance to multiple antibiotics, including quinolones, cephalosporins, ampicillin, nalidixic acid, tetracycline and chloramphenicol[22]. Aspirin could induce multiple antibiotic resistance (*mar*) gene, alter the expression of OMPs, and decrease the outer membrane permeability to antimicrobials or increase the efflux of antimicrobials[35-38]. There were three basic uptake systems across the outer membrane[39], namely, uptake of hydrophilic substances through the water-filled channels of porins, uptake of polycations *via* self-promoted uptake at divalent cation binding sites on lipopolysaccharide, and uptake of hydrophobic substances through the outer membrane bilayer. Bacteria could produce many porins, and were able to regulate the relative number of different porins in response to the osmolarity of the surrounding media. At least five porins named HopA, HopB, HopC, HopD and HopE (part of a 32-member family of outer membrane proteins) were present in a single cell of *H pylori*[40,41]. These porins were considered to be associated with antibiotic resistance[40]. On the other hand, some bacteria expressed a membrane transporter system that led to multidrug resistance by drug efflux. Three putative RND efflux systems, HefABC, HefDEF and HefGHI, identified in *H pylori* may be correlated with antibiotic resistance[42]. Of the three efflux systems, only HefABC was involved in multidrug resistance *in vitro*[42]. Therefore, we tested the five porin genes (*hopA*, *hopB*, *hopC*, *hopD* and *hopE*) and the efflux protein genes (*hefA*, *hefB*, *hefC*) using real-time quantitative PCR, and found that aspirin did not interfere with the expression of the above proteins at the levels of gene transcription.

The alteration of the permeability of outer membrane of *H pylori* should be accompanied by the modification of some related OMPs. In the present study, the expression of two OMPs of *H pylori* between 55 kDa and 72 kDa altered in the presence of aspirin. However, the functions and identifications of these OMPs need to be determined by two-dimensional electrophoresis and protein mass-spectrum analysis. If these OMPs were associated with the increase of the permeability of outer membrane of *H pylori*, further studies should be performed to determine whether the functional and phenotypic alterations of these OMPs in the presence of aspirin occurred at the levels of protein translation or modification, or some other porins or efflux systems were involved.

Park *et al*[43] conducted a pilot study aimed at comparing the efficacy of the standard omeprazole-amoxicillin-clarithromycin (OAC) regimen with a combined OAC regimen and aspirin (OACA). Follow-up endoscopic findings showed that the previous ulcers were completely healed in all subjects. Although the eradication rate for the OACA group (86.7%) was higher than that of the OAC group (80.3%), there was no statistically significant difference between the two groups. The overall adverse events were similar in the two groups. The OACA regimen was well tolerated in the group of patients with peptic ulcer disease. The potential of aspirin and other NSAIDs for clinical use to augment the efficacy of *H pylori* eradication may warrant further investigations.

With the increasing attention paid to the detriment of *H pylori* and the resistance of antimicrobials to this microorganism, it is urgent to investigate new effective therapeutic regimens. Investigating the molecule

mechanisms of aspirin increasing the susceptibility of *H pylori* to antimicrobials will help discover a more effective eradication regimen in clinical practice.

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COMMENTS

Background

It was reported that aspirin inhibited the growth of *Helicobacter pylori* (*H pylori*) and the minimal inhibitory concentration (MICs) of clarithromycin, metronidazole and amoxicillin to *H pylori* decreased when treated with aspirin. This indicated that aspirin increased the susceptibility of *H pylori* to these antimicrobials, and even converted some resistant strains to susceptible ones.

Research frontiers

H pylori infection results in chronic inflammation of gastric mucosa, peptic ulcer and is strongly associated with adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. Recent research revealed that *H pylori* infection played important roles in the invasion of heart and brain vascular disorders, autoimmune diseases, nutritional and metabolic diseases, hematopathy and dermatologic diseases. Eradication of *H pylori* infection is, therefore, very important to prevent and cure these diseases. However, with the wide use of antimicrobials in clinical practice, antibiotic resistance has become apparent and is considered one of the major causes of treatment failure.

Innovations and breakthroughs

In vitro, aspirin decreased the MICs of metronidazole, clarithromycin and amoxicillin to *H pylori*, and even converted some resistant strains to susceptible ones. This study investigated the mechanisms of aspirin increasing the susceptibility of *H pylori* to metronidazole.

Applications

Investigating the molecule mechanisms of aspirin increasing the susceptibility of *H pylori* to antimicrobials will help understand the mechanisms of the resistance of *H pylori* to antibiotics more intensively and discover a more effective eradication regimen in clinical practice.

Terminology

Carbonyl cyanide m-chlorophenylhydrazone (CCCP), a kind of efflux pump inhibitor that is effective at a micromolar concentration, can alter the pH gradient across the cytoplasmic membrane, therefore, deprives the energy provision of the transport protein.

Peer review

The authors intensively reported that non-steroidal anti-inflammatory drugs (NSAIDs), including sodium salicylate, aspirin, indomethacin and celecoxib, inhibited the growth of *H pylori* in a dose-dependent manner and changed the susceptibility of *H pylori* to antibiotics. In this study, the authors demonstrated that although aspirin increased the susceptibility of *H pylori* to metronidazole, it had no effect on the mutations of *rdxA* gene of *H pylori* and that aspirin increased endocellular concentrations of antimicrobials probably by altering the outer membrane proteins (OMPs) expression of *H pylori*. This theme is interesting, and will give new insights of *H pylori* eradication for physicians.

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Hospitalized ulcerative colitis patients have an elevated risk of thromboembolic events

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Abstract

AIM: To compare thromboembolism rates between hospitalized patients with a diagnosis of ulcerative colitis and other hospitalized patients at high risk for thromboembolism. To compare thromboembolism rates between patients with ulcerative colitis undergoing a colorectal operation and other patients undergoing colorectal operations.

METHODS: Data from the National Hospital Discharge Survey was used to compare thromboembolism rates between (1) hospitalized patients with a discharge diagnosis of ulcerative colitis and those with diverticulitis or acute respiratory failure, and (2) hospitalized patients with a discharge diagnosis of ulcerative colitis who underwent colectomy and those with diverticulitis or colorectal cancer who underwent colorectal operations.

RESULTS: Patients diagnosed with ulcerative colitis had similar or higher rates of combined venous thromboembolism (2.03%) than their counterparts with diverticulitis (0.76%) or respiratory failure (1.99%), despite the overall greater prevalence of thromboembolic risk factors in the latter groups. Discharged patients with colitis that were treated surgically did not have significantly different rates of venous or arterial thromboembolism than those with surgery for diverticulitis or colorectal cancer.

CONCLUSION: Patients with ulcerative colitis who do not undergo an operation during their hospitalization have similar or higher rates of thromboembolism than other medical patients who are considered to be high risk for thromboembolism.

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Key words: Ulcerative colitis; Thromboembolism; Hospitalized patients

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INTRODUCTION

Thromboembolic events are a preventable cause of morbidity and mortality in hospitalized patients. In community-based studies, the incidence of thromboembolism in the general population was as high as 1 in 1000[1], depending on age and other risk factors. Hospitalized patients are at increased risk for these events given their acute illness and prolonged immobility.

People with inflammatory bowel disease (IBD) are considered to be at higher risk for thromboembolism than the average population, but the extent of this risk in hospitalized patients with ulcerative colitis (UC) is not well described. In a population-based study, the incidence rate ratio of deep venous thrombosis and pulmonary embolism in people with ulcerative colitis was 3.04[2]. Published venous thromboembolism rates among series of clinic-based patients with ulcerative colitis range from 1.3% to 6.2%[3,4]. In one review of 7199 patients with IBD (half of whom had UC), over the course of 11 years, 1.3% had a thromboembolic event. The mortality of those who had thromboembolism was 25% during the acute thrombotic event[4]. This emphasizes the importance of understanding the risk for thromboembolism in this group of patients and the need

to identify those who would benefit from pharmacologic prophylaxis.

The purpose of our study is to describe the prevalence of thromboembolic events among hospitalized patients with UC, and to compare this to the rate in other hospitalized patients who are considered to be at high risk for thromboembolism.

MATERIALS AND METHODS

Data source

We examined the National Hospital Discharge Survey (NHDS) from years 1979 through 2003[5]. This dataset is compiled by the National Center for Health Statistics and is a probability sample of discharges from short-stay hospitals (average length of stay under 30 d). These data are available on compact disc. The current sampling plan is three-staged. Geographic areas are the primary sampling units, with hospitals selected from within these areas. A sample of discharges from each hospital is then selected by systematic random sampling. Each observation has an associated sample weight that is used to calculate the number of discharges represented by a single observation. Diagnosis and procedure-related information extracted from hospital discharge summaries is coded according to the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9 CM)[6]. Data available for each entry includes demographic information and a maximum of seven ICD-9 CM diagnosis codes and four ICD-9 CM procedure codes. The ICD-9 CM codes are provided in the dataset in the same order as found on the patient discharge summary from which data is abstracted, although it is not confirmed that the first listed code is necessarily the diagnosis on admission. Additionally, if a myocardial event has occurred during the hospitalization, this is automatically listed as the first ICD-9 CM code regardless of the diagnosis on admission.

These de-identified data are publicly available, therefore, the study was acknowledged to be exempt from review by our institutional review board.

Identification of cases

Cases included observations of all ages with an ICD-9 CM code for UC (ICD-9 CM code 556, 556.0-556.9). An ICD-9 CM code for UC was either the first diagnosis (possible primary diagnosis) or any of the other six possible diagnoses, as is true for all other diagnoses examined in this study. Any observation with a code for UC was placed in the appropriate non-surgical or surgical UC group, regardless of the presence of other diagnoses. For observations in which the code for UC was not in the first position, common first-listed diagnoses included gastroenterological conditions such as colitis or gastrointestinal bleeding or abdominal pain; anemia; and volume depletion, all of which could be directly related to UC. Common non-GI first-listed diagnoses included myocardial infarction, stroke, and pneumonia.

The non-surgical comparison groups included all observations with a code for diverticulitis (ICD-9 CM code 562.11 or 562.13) or acute respiratory failure (ICD-9 CM code 518.81, 518.82, 518.84). The observations with acute respiratory failure that also had a procedure code for intubation (ICD-9 CM procedure code 960.4 or 960.5) were excluded as this identified a group of patients who were potentially more sick and had greater risk factors for thrombosis, including prolonged immobility. Observations with diverticulitis were selected as a comparison group because they also had an inflammatory colorectal condition, although the process is localized as opposed to systemic as in UC. Observations with respiratory failure were selected as another comparison group because these are non-surgical patients considered to be at high risk for venous thromboembolic events[7] and are recommended to receive routine thromboembolic prophylaxis when admitted to the hospital. The surgical groups included any observations with UC, diverticulitis, or colorectal cancer (ICD-9 CM code 153, 153.0-153.9, 154, 154.0, 154.1), which also had a procedure code for any colorectal operation (ICD-9 CM procedure code 457, 457.1-457.9, 458, 484, 484.1, 484.9, 485, 486, 486.2-486.9, 461.0, 461.1, 461.3).

We examined both venous and arterial thrombo-embolic events. The two ICD-9 CM codes for pulmonary embolism were 415.1 and 415.19. For deep venous thrombosis, the ICD-9 CM codes included were 451.1, 451.11, 451.19, 451.2, 451.8, 451.81, 451.83, 451.84, 451.89, 451.9, 453.2, 453.8, and 453.9. There were no pregnancy-related thromboembolic events, which carry their own ICD-9 CM codes, among any of the observations examined. Portal vein thrombosis (ICD-9 CM code 452) and renal vein thrombosis (ICD-9 CM code 453.3) were included. There is no code exclusive to mesenteric vein thrombosis, so while this is a described entity in those with UC, it was not examined. Arterial events included aortic or large artery thromboemboli (ICD-9 CM code 444, 444.0, 444.1, 444.2, 444.8, 444.9) and cerebral embolic events (i.e. strokes) (ICD-9 CM code 434.0, 434.1, 434.9).

Covariates examined included age, gender, obesity (ICD-9 CM code 278), atrial fibrillation (ICD-9 CM code 427.31), and prior history of venous thromboembolism (ICD-9 CM code V125.1), which were factors available in this dataset that may affect the risk of thromboembolism or result in a patient being on anticoagulant medications[8,9].

Data analysis

For analyses of the six groups, weights provided with the NHDS dataset were used to estimate population means and proportions. Thus, we first applied probability weights to the sample to calculate the number of discharges represented by each observation in the dataset, and then calculated our prevalence rates. These six groups are, therefore, referred to as discharges, grouped by the various conditions previously mentioned. Information on sampling strata and primary sampling units is not provided with the NHDS dataset,

Table 1 Characteristics of non-surgical and surgical groups

	Non-surgical discharges			Surgical discharges		
	Ulcerative colitis	Diverticulitis	Acute respiratory failure	Ulcerative colitis	Diverticulitis	Colorectal cancer
N	7302	25138	50905	880	6860	20336
Estimated population totals[1]	974206	3805999	6296383	127327	944275	2910807
Age (mean)	49.8	67.7	62.3	47.6	62.2	69.6
Male (%)	46	35[a]	48	54	43	49
Length of stay (mean)	7.1[d]	6.6[d]	11.7[d]	16.9[d]	13.1[d]	13.9[d]
Atrial fibrillation (%)	2.9	4.5[a]	11.3[c]	2.3	3.2	5.1[e]
Obesity (%)	1.3	2.5[a]	2.2[c]	1.1	2.3	1.5
History of VTE (%)	0.13	0.01	0.11	0	0.06	0.18
Mortality (%)	1.6	1.6	23.6	2.5	4.0	3.7

VTE: Venous thromboembolism. [1]Calculated by applying probability weight to each observation. [a]$P < 0.05$, non-surgical discharges with ulcerative colitis *vs* non-surgical discharges with diverticulitis; [c]$P < 0.05$, non-surgical discharges with ulcerative colitis *vs* non-surgical discharges with respiratory failure; [e]$P < 0.05$, surgical discharges with ulcerative colitis *vs* surgical discharges with colorectal cancer.

so confidence intervals and *P*-values based on the linearization methods appropriate for complex surveys could not be computed. Instead, standard errors and confidence intervals for estimated proportions were approximated using the methods and constants provided in the NHDS documentation[5]. Approximate two-sided *Z*-tests were then used to evaluate differences in rates between discharges with UC and each comparison group, under the assumption of negligible correlation between the group-specific estimates. Differences in gender and prevalence of atrial fibrillation, obesity, and history of venous thromboembolic events were also analyzed using the *Z*-test. We also calculated the mean age of the observations in each group.

We then performed three further sets of analyses. In the first, we compared event rates among the non-surgical discharges with UC, diverticulitis, or acute respiratory failure. In the second, we compared event rates among surgical discharges with UC, diverticulitis, or colorectal cancer. Finally, we compared event rates between surgical discharges with UC and non-surgical discharges with UC.

Logistic regression was performed to assess the degree to which between-group differences in event rates might be explained by potential confounders including age, gender, obesity, and atrial fibrillation. Weighted odds-ratios for the association of patient group with event risk can be validly estimated using these data under the working assumption of independence, as in the standard linearization procedures for logistic regression analysis of complex survey data. We defined these regression analyses as exploratory analyses because no valid confidence intervals or *P*-values can be computed because of the lack of dataset-specific information on sampling stratum and primary sampling units. Despite this limitation, some information is gained by comparing the unadjusted and adjusted odds ratios in cases where the rate difference corresponding to the unadjusted odds ratio is statistically significant. If the adjusted odds ratio is farther from the null value of 1.0 than the unadjusted odds ratio, then it is plausible that the adjusted odds ratio is also statistically significant.

RESULTS

Patient characteristics

We examined 8182 observations with UC, 89.2% of whom had no concurrent colorectal operation during the admission. This group was compared with 25138 observations with diverticulitis who were not treated surgically and 50905 observations with respiratory failure. The 880 observations with UC who were treated surgically were compared with 6860 observations with surgically treated diverticulitis and 20336 observations with surgically treated colorectal cancer. The estimated population totals after application of the sampling weights and the characteristics of the non-surgical and the surgical groups are presented in Table 1.

Non-surgical discharges

Discharges with UC who did not have surgery had a deep venous thrombosis rate of 1.62% (95% CI, 1.01%-2.23%), a pulmonary embolism rate of 0.51% (95% CI 0.25-0.78%), and a combined venous thromboembolic event rate of 2.03% (95% CI, 1.36%-2.69%; Figure 1). The rates of deep venous thrombosis ($P = 0.01$), pulmonary embolism ($P = 0.03$), and combined venous thromboembolic events ($P = 0.004$; defined as deep venous thrombosis, pulmonary embolism, portal and renal vein thromboses) among those with UC were significantly higher than those for discharges with diverticulitis (Table 2). Discharges with UC had similar rates of deep venous thrombosis and combined venous thromboembolic events and lower rates of pulmonary embolism ($P = 0.002$), strokes ($P < 0.001$), and arterial embolisms ($P = 0.01$) than discharges with respiratory failure (Table 2). The rates for portal vein thrombosis and renal vein thrombosis were very low in non-surgical discharges with UC (0.05% and 0%, respectively) and not significantly different from rates in the comparison groups.

In an exploratory analysis, odds ratios for the non-surgical discharge groups were compared before and after adjustment for age, gender, obesity, and atrial fibrillation. For the comparison of UC to diverticulitis, the odds ratios increased after adjustment for potential confounders

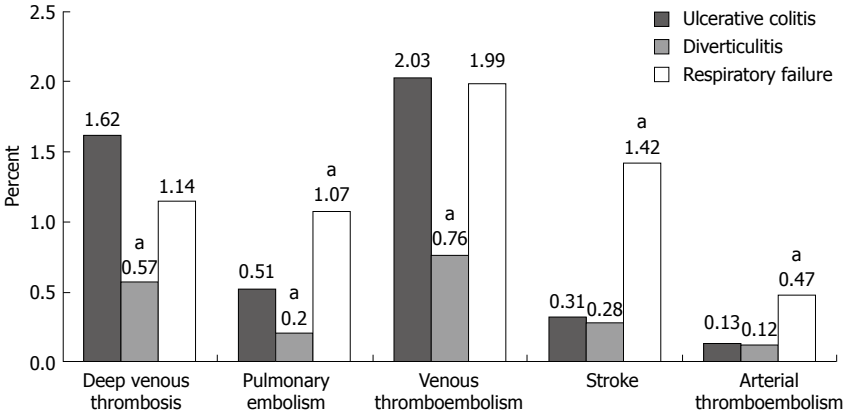

Figure 1 Rates of different thromboembolic events in non-surgical comparison groups. Confidence intervals for the individual rates are shown in Table 2. [a]$P < 0.05$, *vs* the ulcerative colitis discharges.

Table 2 Estimated population percentages of non-surgical discharges which had thromboembolic events and comparison of event rates

Thromboembolic event	Ulcerative colitis *vs* diverticulitis		Ulcerative colitis *vs* respiratory failure	
	Rate difference, % (95% CI)	*P* value	Rate difference, % (95% CI)	*P* value
Deep venous thrombosis	1.05 (1.69 to 0.41)	0.01	0.48 (-0.18 to 1.14)	0.2
Pulmonary embolism	0.31 (0.03 to 0.59)	0.03	-0.56 (-0.92 to -0.2)	0.002
Venous thrombo-embolism	1.26 (0.56 to 1.97)	0.004	0.04 (-0.72 to 0.8)	0.9
Stroke	0.03 (-0.28 to 0.34)	0.9	-1.11 (-1.52 to -0.7)	< 0.001
Arterial thrombo-embolism	0.01 (-0.21 to 0.24)	0.9	-0.33 (-0.59 to -0.08)	0.01

from 2.69 to 3.05 for combined venous thromboembolic events (CI's not calculable; see methods). Similarly, the odds ratios for UC compared to acute respiratory failure increased after adjustment, from 1.02 to 1.12 for combined venous thromboembolic events. The increase in odds ratios after adjustment supports the view that the associations observed on univariate analysis are, in fact, significant. If anything, the association is further strengthened (with the odds ratio moving farther away from 1.00) by adjusting for the potential confounders. When comparing odds of arterial embolism, the odds ratio for UC compared to diverticulitis increased from 1.12 to 1.48 and the odds ratio for UC compared to respiratory failure increased from 0.29 to 0.39.

Surgical discharges

Discharges with UC who underwent an operation for this condition had a deep venous thrombosis rate of 1.11% (95% CI, 0%-2.76%), a pulmonary embolism rate of 0.13% (95% CI, 0%-0.6%), and a combined venous thromboembolism rate of 1.20% (95% CI, 0%-2.89%; Figure 2). The rates of these venous thromboembolic events did not significantly differ among the three surgical groups (Table 3). Discharges with UC had a portal vein thrombosis rate of 0.02% and no occurrence of renal vein thromboses. These event rates were similarly low in the surgical comparison groups. The rates of stroke or other arterial embolic events did not differ among the three surgical groups (Table 3).

An exploratory analysis of the role of confounding variables for surgical discharges was also performed. For the comparison of surgical UC and diverticulitis discharges, the odds ratios increased after adjustment for potential confounders from 2.76 to 3.47 for deep venous thrombosis (CI's not calculable, see methods), from 1.40 to 1.74 for combined venous thromboembolic events, and from 1.28 to 1.93 for arterial embolism. When discharges with UC and surgery were compared with discharges with colorectal cancer and surgery, the odds-ratios increased after adjustment from 1.62 to 2.17 for deep venous thrombosis, from 1.00 to 1.40 for combined venous thromboembolic events, and from 1.62 to 3.95 for arterial embolism. Once again, the increase in odds ratios after adjustment supports the significance of the associations found in the univariate analysis. For stroke, the adjusted odds reversed in both comparison groups such that after adjustment, the discharges with UC had greater odds of stroke than discharges with diverticulitis, changing from 0.97 to 1.60, than discharges with colorectal cancer, changing from 0.67 to 1.50.

Comparison of discharges with UC who had surgery and those who did not

Although the rates of deep venous thrombosis, pulmonary embolism, combined venous thromboembolic events, and stroke were higher in discharges with UC who did not have surgery than in those who did, the differences were not statistically significant (Figure 3).

Mortality

Among discharges with UC who did not have surgery, mortality was higher for those who had a venous thromboembolic event than in those who did not, although the difference was not statistically significant (5.5% *vs* 1.5%, $P = 0.1$). Similarly, mortality was higher from arterial events (17.5% *vs* 1.6%; *P* value, not calculable due to missing events). Among discharges

Table 3 Estimated population percentages of surgical discharges with thromboembolic events and comparison of event rates

Thromboembolic event	Ulcerative colitis *vs* diverticulitis		Ulcerative colitis *vs* respiratory failure	
	Rate difference, % (95% CI)	*P* value	Rate difference, % (95% CI)	*P* value
Deep venous thrombosis	0.7 (-0.98 to 2.39)	0.4	0.42 (-1.25 to 2.1)	0.6
Pulmonary embolism	-0.36 (-0.95 to 0.23)	0.2	-0.42 (-0.92 to 0.09)	0.1
Venous thrombo-embolism	0.34 (-1.42 to 2.09)	0.7	0.01 (-1.61 to 1.72)	1.0
Stroke	-0.01 (-0.67 to 0.65)	1.0	-0.14 (-0.75 to 0.48)	0.7
Arterial thrombo-embolism	0.04[1]	-[2]	0.06[1]	-[2]

[1]Confidence interval not available because there were no events after 1988; [2]*P* value not calculable because there were no events in the ulcerative colitis group after 1988.

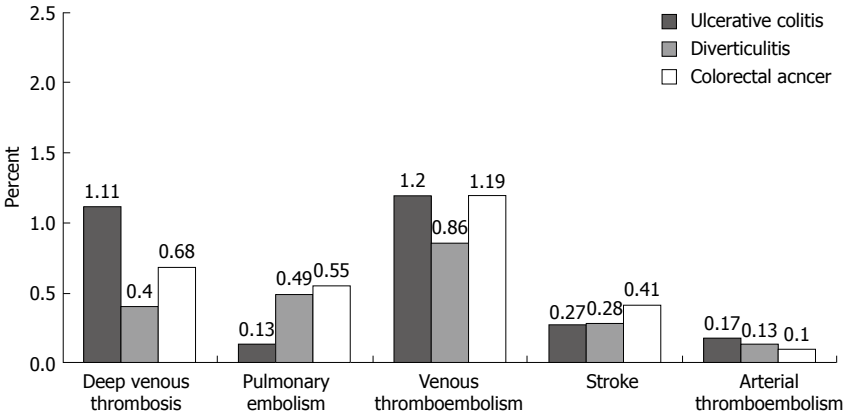

Figure 2 Rates of different thromboembolic events in surgical comparison groups. Confidence intervals for the individual rates are shown in Table 3.

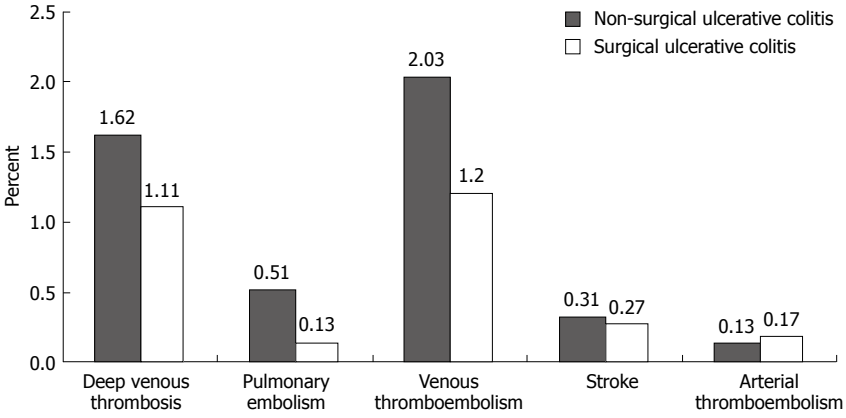

Figure 3 Rates of different thromboembolic events in non-surgical and surgical discharges with ulcerative colitis. Refer to Tables 2 and 3 for confidence intervals for the individual rates displayed above.

with UC who did have surgery, mortality was likewise higher in those who had a venous thromboembolism (5% *vs* 2.5%, $P = 0.9$) or arterial thromboembolism (0 *vs* 2.5%; *P* value, not calculable due to missing events) than those who did not, although the differences were not statistically significant.

DISCUSSION

In this study, we compared the prevalence rates of venous and arterial thromboembolic events in hospitalized patients with a diagnosis of UC to those of other high-risk hospitalized patients, using a national discharge dataset. The confidence intervals and *P*-values we calculated in the unadjusted analysis summarize the evidence for or against between-group differences in crude event rates. We found that discharges with UC who did not have surgery had a higher rate of deep venous thrombosis and pulmonary embolism than discharges with diverticulitis treated non-operatively, a group that had a greater prevalence of risk factors for thromboembolism, such as older age and obesity. We also found that compared to discharges with respiratory failure, the discharges with UC who did not have surgery had a similar rate of deep venous thrombosis, but a lower rate of pulmonary embolism. The higher rate of pulmonary embolism in discharges with acute respiratory failure could be related to two factors. It might be due to a causal relationship between pulmonary embolism and acute respiratory failure. On the other hand, the higher rate could result from ascertainment bias, with a higher rate of diagnosis of pulmonary embolism in patients with respiratory failure and pulmonary symptoms because more pulmonary imaging was performed on these patients.

Among surgical discharges with UC, diverticulitis, or

colorectal cancer, there were no differences in rates of deep venous thrombosis or pulmonary embolism. We hypothesize that this could be explained by the frequent use of thromboembolic prophylaxis in inpatients undergoing a colorectal operation, which would minimize differences in their risks of thromboembolism. However, this cannot be confirmed, because the dataset does not provide information about individual medications. We also found no difference in thromboembolic event rates between non-surgical and surgical discharges with UC. One may think that postoperative patients would be less mobile than non-surgical patients, an additional risk factor for thromboembolic events. However, the use of thromboembolic prophylaxis may mask any difference between the surgical and non-surgical groups.

The exploratory logistic regression analyses, while not definitive, strongly suggest that the statistically significant higher event rates that we found among both surgical and non-surgical discharges with UC in the unadjusted analysis were not meaningfully confounded by between-group differences in age, gender, obesity, atrial fibrillation, or prior history of venous thromboembolism.

No prior publication has examined the incidence of thromboembolism in patients with UC specifically during hospitalization, although several series have reviewed an institution's experience with IBD and thromboembolism. One very large single-center study of patients with UC and Crohn's disease, which reported a 1.3% incidence of thromboembolic events, found that 64% of patients with an event had active disease, 26% had disease controlled by a sulfasalazine or corticosteroid, and 10% were in remission[4]. A population-based study in Manitoba using administrative data demonstrated that patients with Crohn's disease and those with UC had approximately three times the risk of developing deep venous thrombosis or pulmonary embolism compared to controls from the general population, matched for year of diagnosis, age, gender, and area of residence[2]. This study also attempted to determine whether thromboembolic risk was higher in patients with UC because of frequent hospitalization, but concluded that this was not the case because the rate ratio among only hospitalized patients was similar to that of the entire population. Another study compared the prevalence of venous thromboembolism between outpatients with IBD and matched healthy controls and found that patients with IBD had greater odds of thromboembolism (OR = 3.6; 95% CI, 1.7-7.8; adjusted for operation, injuries, oral contraceptive use, pregnancy, body mass index, and smoking)[3].

Other studies have focused on specific types of venous thromboembolic events in patients with IBD[10,11]. In a review of 94 patients who underwent restorative proctocolectomy and had a postoperative computed tomography scan, 45% had portal vein thrombosis[10]. The portal vein thrombosis rate in the patients with UC who underwent colorectal surgery in our study was only 0.02%. Not all of the patients underwent a total colectomy, which is hypothesized as the main risk factor for portal vein thrombosis. In addition, we cannot be certain if the low rate we found is due to the low sensitivity of ICD-9 CM coding for portal vein thrombosis or, more likely, the fact that most patients with UC were not assessed for the occurrence of this condition. A separate study examining superior mesenteric vein thrombosis after colectomy described that 4.8% of the 83 patients who underwent colectomy for IBD developed CT-proven superior mesenteric vein thrombosis[11]. We did not examine mesenteric venous thrombosis because of the unreliability of ICD-9 CM coding for this diagnosis. Arterial thromboembolic events in patients with UC have also been described, with documentation of these events in several case studies and one study of a cohort of IBD patients describing peripheral arterial thrombosis and cerebrovascular accidents in 0.002%[4,12,13]. The magnitude of the risk for arterial events compared with that for the general population is not known.

The reason for the increased rate of thromboembolic events in patients with UC remains uncertain, but most likely is related to the interaction between cytokine mediators of chronic inflammation and the coagulation cascade[14]. No study has convincingly demonstrated that patients with IBD have a greater burden of prothrombotic risk factors than the general population, such as factor V Leiden mutations[15], hyperhomocysteinemia[16], antiphospholipid antibodies, or thrombophilia[17,18]. However, two studies did identify a higher prevalence of factor V Leiden mutations in patients with IBD who developed thromboembolism than in those who did not[15,19].

Does the demonstration that hospitalized patients with UC are at increased risk for thromboembolic events, especially venous thromboembolism, merit a recommendation that these patients receive thromboembolic prophylaxis? To answer this question, one must consider the possible effects of the prophylaxis and balance its risks with the benefits of preventing thromboembolism. The bleeding risks unique to UC patients from pharmacologic heparin therapy can be explored in studies of heparin for treating active UC. Two randomized controlled trials have evaluated unfractionated heparin. In one study of patients with UC, all of whom had rectal bleeding, three of the 12 patients receiving full anticoagulation with heparin developed increased rectal bleeding, one requiring an urgent operation[20]. In the other study, of eight patients with IBD who received a continuous infusion of unfractionated heparin, no major bleeding events occurred[21]. Other trials have examined treatment with low molecular weight heparin. In one randomized trial including only patients with UC, the 16 patients who received full anticoagulant doses of low molecular weight heparin had no episodes of rectal bleeding and only small hematomas at the injection site[22]. In a larger, randomized, controlled trial of 48 patients with UC who received low molecular weight heparin, first at full

anticoagulation doses, and later at a dose equivalent to that used for prevention of deep venous thrombosis, there were no complications in the treatment group and one episode of rectal bleeding in the placebo group[23]. Overall, it appears that heparin therapy at doses sufficient to achieve complete anticoagulation is safe among patients with active UC with respect to GI bleeding risks, and that the lower thromboembolic prophylactic doses are likely to not bear any higher risk.

At the same time, the consequences of thromboembolism in patients with UC appear to be serious, as one study reported that 25% of IBD patients who developed a thromboembolism died from related events[4]. In our dataset, non-surgical patients with UC who had either a venous or arterial thromboembolism had a mortality rate of 5.5% and 17.5%, respectively, compared to a mortality of 1.5% and 1.6%, respectively in non-surgical patients with UC with no thromboembolic events, though we were unable to determine specifically to what extent thromboembolism contributed to the individual patients' death. Few studies have specifically examined an inpatient population, so the effects of a thromboembolic event on other important outcomes, such as length of stay and cost, have not been described. In studies of other high risk patient populations, prophylaxis reduces morbidity and mortality in medical patients[24]. Thus, given the higher mortality rate in UC patients with thromboembolism and the low risk of heparin therapy in UC patients, it appears that thromboembolic prophylaxis might be justified. Of course, another important aspect of caring for hospitalized IBD patients is rapid, optimal control of the active disease, as there are studies that suggest that those with active or more severe disease have a greater risk for thromboembolism[3,4,25,26]. We were unable to determine the severity of disease in the patients in this dataset.

The primary strength of our study was the ability to examine thromboembolic events across a large, diverse population from a representative national sample of hospitalized patients. The NHDS dataset is an excellent resource because it captures a broad spectrum of patients. However, because the dataset is based on ICD-9 CM coding, there is the potential for diagnostic misclassification. While ICD-9 CM coding is generally considered to be highly specific and have a low false-positive rate, coding can also have low sensitivity, resulting in omission of cases[27]. This would imply that our calculated event rates are lower than actual rates, although this misclassification should not differ among the patient groups examined; therefore, our rate comparisons would still be valid. The NHDS dataset has been well-described for examining trends of thromboembolic disease in various risk groups, and conclusions drawn from the relationship of venous thromboembolism to age, race, obesity, and cancer are supported by other studies of those patient populations using different data sources[28-31]. For example, one study examined differences in venous thromboembolic event rates with respect to age in the NHDS dataset. The rate of venous thromboembolism increased in relation to older age, a finding which is supported by other regional population-based studies[30]. In addition, when the NHDS dataset was used to examine stroke as a predictor of venous thromboembolic events, the rates were similar to that in a prospective trial, thus suggesting that the coding for diagnosis of stroke was adequate[32]. Therefore, while the diagnosis and procedure codes in the NHDS have not been validated, findings based on its data reflect those of other data sources.

The NHDS dataset we used enabled us to perform a cross-sectional analysis of the prevalence of thromboembolic events in hospitalized patients. However, a major limitation of our study is that there are covariates known to be associated with thromboembolism which could not be examined because they were not provided in the dataset, such as severity of UC, medications (e.g. steroids, thromboembolic prophylaxis or anticoagulation, oral contraceptives), tobacco use, actual timing of the event, and reason for hospitalization. We cannot distinguish whether the thrombosis was perhaps the reason for admission as opposed to an event that happened during the hospitalization. In addition, we don't know if UC was active and the reason for admission, or whether it was merely a part of the past medical history for that observation. Fifty percent of the non-surgical UC group did have UC as the first-listed ICD-9 CM code, and many of the other common first-listed diagnoses were gastroenterology-related, but our data is limited in that we cannot verify the effect of active UC on TE risk. Eighty percent of the surgical UC group had UC as the first-listed ICD-9 CM code, which is reasonable because those undergoing an operation during that admission would be likely to have their primary diagnosis be UC. We acknowledge there may be bias in that some observations might have more active disease than others, but this study is a broad examination of trends in TE rates across hospitalized patients with a history of UC and does not attempt to stratify TE risk under specific conditions.

Given that the NHDS samples discharges and not individual persons, we acknowledge the possibility that a person with several admissions for the same problem could be sampled more than once. Another limitation is that the NHDS does not provide the information required for valid calculation of standard errors in a logistic regression. Thus, whether or not the thromboembolic rates would differ between discharges with UC and other high-risk discharges after adjustment for potential confounding cannot be determined. However, the significantly higher thromboembolism rates among non-surgical discharges with UC compared to high-risk discharges with diverticulitis in unadjusted analysis, and the fact that adjustment increased the odds ratios for UC in the logistic model, strongly suggest that observed differences in thromboembolic event rates are independent of potential confounding effects.

In spite of these limitations, our findings suggest that in hospitalized patients with UC, the risk for thromboembolic events is as great or greater than for other hospitalized medical patients who are considered

to have a high risk for thromboembolism, and for whom thromboembolic prophylaxis is recommended routinely. These data highlight the importance of further prospective studies to clarify the risks and benefits of thromboembolic prophylaxis in patients with UC and to establish the optimal prophylactic regimen.

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COMMENTS

Background

People with inflammatory bowel disease (IBD) are considered to be at higher risk for thromboembolism than the average population, with up to three times the incidence rate of events compared with those without IBD. Hospitalized patients may be at an additional increased risk of thromboembolism given their decreased mobility or postoperative state. However, the rate of thromboembolic events in hospitalized IBD patients is not well described, and could have bearings on the degree of prophylaxis recommended for these patients.

Research frontiers

Important areas of research surround studies of the prevalence of prothrombotic factors such as homocysteine, activated Protein C resistance, and gene mutations in the susceptibility of IBD patients to thromboembolic events. Larger studies need to be conducted to determine whether the prevalence of such disorders is truly higher in IBD, and thus if detected can help stratify those who need more vigilance for thromboembolic events.

Innovations and breakthroughs

Few studies have examined the rate of thromboembolic events among a nationally representative hospitalized population. We confirmed previous evidence that ulcerative colitis (UC) patients have a higher rate of thromboembolism than other groups of patients, in particular those groups of patients also considered to be at higher risk.

Applications

This study emphasizes that among hospitalized patients considered to be at risk for thromboembolic events, those with UC appear to have a higher rate of thromboembolism. This can have a bearing on the degree of prophylaxis for DVT/PE that these patients receive. In addition, if patients are identified to be at higher risk there may be those that benefit to prophylaxis even when not hospitalized.

Peer review

This manuscript is reasonably well done, but does not add much to our knowledge about thromboembolism in IBD.

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BRIEF ARTICLES

Esophageal cell proliferation in gastroesophageal reflux disease: Clinical-morphological data before and after pantoprazole

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Abstract

AIM: To evaluate esophageal mucosal defense mechanisms at an epithelial level to establish if pantoprazole treatment can induce ultrastructural healing and improvement in the proliferation activity of the esophageal epithelium in gastroesophageal reflux disease (GERD).

METHODS: This was a single-blinded study for pH-monitoring, and histological, ultrastructural and MIB1 immunostaining evaluation. Fifty eight patients with GERD were enrolled and underwent 24 h pH-monitoring and endoscopy. Patients were treated for 12 and 24 mo with pantoprazole. Esophageal specimens were taken for histological and ultrastructural evaluation, before and after the treatment.

RESULTS: With transmission electron microscopy, all patients with GERD showed ultrastructural signs of damage with dilation of intercellular spaces (DIS). After 3 mo of therapy the mean DIS values showed a significant reduction and the mean MIB1-LI values of GERD showed an increase in cell proliferation. A further 3 mo of therapy significantly increased cell proliferation only in the erosive esophagitis (ERD) group.

CONCLUSION: Three months of pantoprazole therapy induced ultrastructural healing of mucosal damage in 89% and 93% of ERD and non-erosion patients, respectively. Moreover, long-term pantoprazole treatment may be helpful in increasing the capability for esophageal cell proliferation in GERD, particularly in ERD patients.

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Key words: Gastroesophageal reflux disease; Esophagitis; Cell proliferation; Electron microscopy; Pantoprazole

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is represented by a broad spectrum of endoscopic and histological features ranging from endoscopically and histologically normal mucosa to severe endoscopic erosive esophagitis (ERD) accompanied by extensive histological abnormalities[1-2]. Acid and bile exposure times in GERD patients who are endoscopically negative (NERD) and in subjects with ERD greatly overlap, as demonstrated by 24-h monitoring methodologies and ultrastructural alterations[3-5]. This strongly implies that there must be other factors that are important in defining the degree of gross and microscopic changes within the esophageal mucosa under the impact of the aggressive milieu of the

gastroesophageal refluxate. Esophageal mucosal defense mechanisms, balancing luminal aggressive factors, operate at three overlapping levels: the pre-epithelial, epithelial and post-epithelial[6-9]. Because the aggressive factors of gastroesophageal reflux always act at the luminal perimeter of the esophageal mucosa, pre-epithelial defense remains a vanguard of mucosal protection.

The importance of the squamous epithelium biopsy in NERD diagnosis has been reviewed in consideration of the recognition of new histological parameters such as intercellular space dilations[10-13].

Recent data about cell kinetics of the esophageal mucosa have shown that in patients affected by GERD, the proliferation rate of the esophageal epithelium was inferior to that of normal subjects. Ki67-LI gives an accurate estimate of the growth fraction and is reduced in esophageal mucosa exposed to chronic acid. In particular, patients with GERD have a decrease in MIB1 immunostaining of 50% and 25% in NERD and ERD compared to normal subjects[14].

The primary goals of GERD therapy are enduring symptom relief, protection from long-term complications and improved subjective well-being. Proton pump inhibitors (PPIs), which provide powerful gastric acid control[15,16], are the treatment of choice in this regard[17,18].

The aim of this study was to evaluate the esophageal mucosal defense mechanisms at the epithelial level. Transmission electron microscopy (TEM) and MIB1 antibodies were used to establish if pantoprazole treatment could induce ultrastructural healing and an improvement in the proliferation activity of the esophageal epithelium in patients with erosive or non-erosive GERD.

MATERIALS AND METHODS

Study design

This study was single-blinded for pH-monitoring and histological, ultrastructural and MIB1 immunostaining evaluations. Patients gave written informed consent to participate in the study, which was approved by the local research committee.

Study populations

We enrolled 58 patients (26 male; mean age 45.22 ± 12.92; range 23-72) with typical symptoms of GERD (heartburn and/or regurgitation) with at least a 1-year history of GERD (with a frequency of more than twice a week). These were consecutive patients who agreed to undergo both esophageal pH-monitoring and endoscopy. The frequency and intensity of symptoms and their impact on the patients' quality of life were registered using a structured and validated questionnaire for the diagnosis of GERD[19], and patients with a score higher than 3.1 were considered positive.

Patients with esophageal or gastric malignancy or histologically-proven Barrett esophagus, gastric or duodenal ulcer, previous esophageal or gastric surgery were excluded. Patients taking antisecretory or prokinetic drugs were asked to stop any medication at least 30 and 15 d before the study, respectively. Antacid or alginate preparations were suggested in case of frequent and intolerable symptoms.

The control group consisted of 9 healthy voluntary subjects (mean age 38.2 ± 17.6 years, range 24-60 years; 4 male), and were defined according to the following parameters: absence of typical symptoms or atypical manifestations of GERD, normal 24-h esophageal pH monitoring, endoscopic and histological features, and ultrastructural parameters.

Twenty-four-hour ambulatory pH monitoring

All patients underwent 24-h esophageal pH-monitoring. During the test, meal times and compositions were standardized. The reflux parameters were assessed according to Johnson-DeMeester[20]. The percentage of time with pH < 4.0 over 24 h was evaluated and was considered abnormal if pH < 4.0 was present for more than 6% of the total 24-h period. In the week after 24-h pH monitoring, all the patients underwent upper-gastrointestinal endoscopy to assess the presence or absence of erosive esophagitis.

Endoscopic evaluation

Patients underwent upper gastrointestinal endoscopy (videogastroscope Olympus GIF 140) after sedation by i.v. administration of midazolam (2.5 mg) to assess the presence or absence of esophagitis. The Los Angeles classification was used to grade the esophagitis[21]. During endoscopy, eight biopsies were taken as follows: two biopsies from each of the four quadrants, 5 cm above the squamo-columnar junction (SCJ), from macroscopically intact (non-eroded) esophageal mucosa. The SCJ (or Z-line) was defined as the border between gastric glandular and esophageal squamous epithelium, and roughly corresponded to the proximal edge of the gastric folds.

Of the 8 specimens taken, 6 were oriented to appropriate cellulose acetate supports (Endofilters Bioptica, Milan, Italy), fixed in 10% buffered formalin and embedded in paraffin, for processing by hematoxylin-eosin and MIB1 evaluation. Two specimens from each patient were used for processing by TEM according to our methodology[11]. Of 58 patients enrolled, 30 patients were affected by NERD (11 male; mean age 49.33 ± 10.19; range 36-61) and 28 by ERD (13 male; mean age 43.93 ± 12.98; range 23-72).

Histological evaluation

Serial sections of 4 μm were cut from each paraffin block and stained with hematoxylin-eosin. For each case, whole longitudinally-sectioned samples were examined. Esophagitis was identified and graded according to the Ismail-Beigi *et al*[1] classification.

MIB1 immunostaining and quantification

MIB1 immunostaining was assessed using anti-Ki-67 monoclonal antibodies (MoAbs) (clone MIB-1; BioGenex Laboratories, San Ramon, CA, USA). Before immunostaining, antigen retrieval was effected by heating the slides, which were fully immersed in 10 mmol/L

sodium citrate buffer (pH 6.0) for 20 min in an autoclave. After cooling to room temperature, the slides were incubated with primary MoAbs overnight at a dilution of 1:100. The immunostaining reaction was then developed according to SABC (stretavidin-biotin-peroxidase preformed complex) protocol and highlighted using a peroxidase/DAB enzymatic reaction. Sections were finally counterstained with hematoxylin[22].

Quantitative analysis of MIB1 immunostaining was performed on a contiguous field, visualized on the color monitor of a Pentium Ⅲ PC equipped with a 3 CCD (charge-couple device) color video camera (KY F55B, JVC, Pinebrook, NJ, USA) and connected to a light microscope (Leitz DIAPLAN). For each case, whole longitudinally-sectioned samples were examined. Samples that did not contain at least 1000 cells were excluded. Quantitative evaluation was only carried out on portions of the epithelium in between vertically-sectioned stromal papillae and corresponding to 100 μm from the basal layer. The MIB1 label index (MIB1-LI) was defined as the ratio of MIB1 positive nuclei to the total number of epithelial cells, and was expressed as a percentage.

Transmission electron microscopy (TEM)

The specimens were rinsed in buffer, post-fixed in 1% buffered osmium tetroxide, and dehydrated through a graded alcohol series. They were then infiltrated through propylene oxide and embedded in an epoxy resin. Blocks were trimmed and ultra-thin sections on copper grids were post-stained with uranyl acetate and lead citrate. Each specimen was analyzed by TEM (Philips 510) and then photographed at an accelerating voltage of 80 kV. Photographs of at least 10 significant fields, each obtained with a negative containing an internal scale marker, were magnified at 3500 ×. Ten photomicrographs were obtained from each patient's biopsy specimens observed by TEM. Photographs with internal scale marker were digitized and then each field was valued using Endox System software. At least 10 randomly selected perpendicular transects to adjacent membranes were drawn and measured in each image for a total of 100 measurements in each case. Each transect was drawn at a distance no closer than 1 μm. A mean score of dilations in the intercellular space (DIS) of 0.74 μm was considered a cut-off score for damage[11].

Treatment

After endoscopy patients were treated with pantoprazole 40 mg/d for 12 wk. After this period they underwent endoscopy and another series of biopsies were taken and processed, as previously described. At the end of these 12 wk, 50% of patients with ERD (n = 14) and 50% of patients with NERD (n = 15) were randomized, using a computer generated list, to receive an additional 12 wk of therapy. After this second period of therapy, the treated patients underwent endoscopy and a final series of biopsies were taken and processed, as previously described. No therapy with pantoprazole was administered in healthy controls.

Statistics

The measurements obtained by the above-mentioned method were used to calculate mean DIS scores for each patient and all cases as a whole. The Student's t-test was performed for both independent variables. The Mann-Whitney U test was performed to compare cell kinetic data in each group of subjects. The paired-sample t-test was performed to compare the means of DIS and MIB1-LI values before and after therapy for each group of patients studied. $P < 0.05$ was considered statistically significant. Data were analyzed with SPSS software (SPSS, Chicago, IL).

RESULTS

The percentage time of esophageal pH < 4 ranged from 8.7% to 12.8% among all patients, with a mean ± SD value of 10.3% ± 1.8% and a median value of 10%. The percentage time of esophageal pH < 4 for the two groups of patients (NERD and ERD) was 10% ± 1.1% and 10.5% ± 1.3%, respectively. No significant differences were found between the two groups.

Among 28 patients affected by ERD, 22 had a normal histological pattern in specimens of endoscopic mucosa, and 6 had mild esophagitis. In the NERD group, 29 patients showed a normal pattern and only one had histological signs of esophagitis (mild).

Using TEM, all patients with GERD, with or without erosions, showed ultrastructural signs of damage defined by the presence of DIS (cut-off of DIS > 0.74 μm) at baseline (T0)[11]. DIS ranged from 0.28 to 7.83 μm among all subjects, with a mean ± SD value of 1.83 ± 0.33 μm and a median value of 2.27 μm. The mean values of DIS in the three groups of subjects (normal, NERD and ERD) were 0.48 ± 0.09, 2.11 ± 0.22 and 2.27 ± 0.48 μm, respectively. The difference between normal and. patient groups was significant ($P < 0.001$), while no significant difference was found in the mean value of DIS between the two GERD groups (NERD *vs* ERD). After 3 mo of therapy, the mean DIS values were 0.55 ± 0.11 and 0.58 ± 0.13 in NERD and ERD patients, respectively. A paired-sample t-test conducted to compare the mean DIS values measured in each patient at T0 and T3, showed a significant reduction of intercellular spaces both in ERD and NERD patients ($P < 0.001$) (Figure 1). Figure 2 shows TEM photomicrographs of the suprabasal layer of esophageal mucosa before (A) and after (B) pantoprazole treatment. The intercellular spaces clearly recovered after therapy.

At baseline (T0), MIB1-LI ranged from 12% to 78.8% among all patients, with a mean ± SD of 32.2% ± 16.3%. The mean MIB1-LI value of the healthy voluntary controls was 65.9% ± 9.6%. In 58 GERD patients, 30 with NERD and 28 with ERD, the mean values of MIB1-LI were 31.3% ± 8.8% and 22.3% ± 7.9%, respectively, with a significant difference between the two groups ($P < 0.001$). In all three groups, proliferating cells were located mainly in the basal zone (100 μm from the basal layer), with no differences in

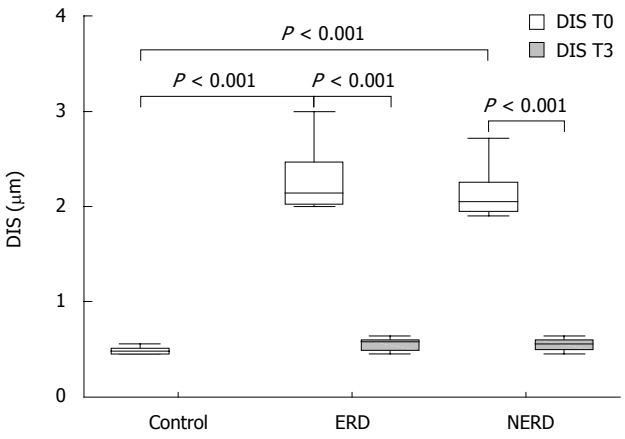

Figure 1 Box-plots of dilation of intercellular spaces (DIS) values, DIS median (bold line in the box), and interquartile range (upper and lower lines of the box) in human esophageal mucosa of healthy controls and ERD and NERD patients at baseline (T0) and after 3 mo of therapy (T3).

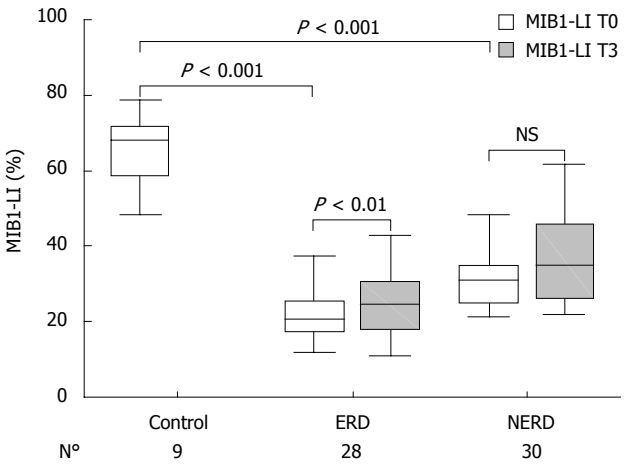

Figure 3 Box plots of MIB1-LI values, LI median (bold line in the box), and interquartile range (upper and lower lines of the box) in human esophageal mucosa of healthy controls and ERD and NERD patients, basal (T0) and after 3 mo of therapy (T3).

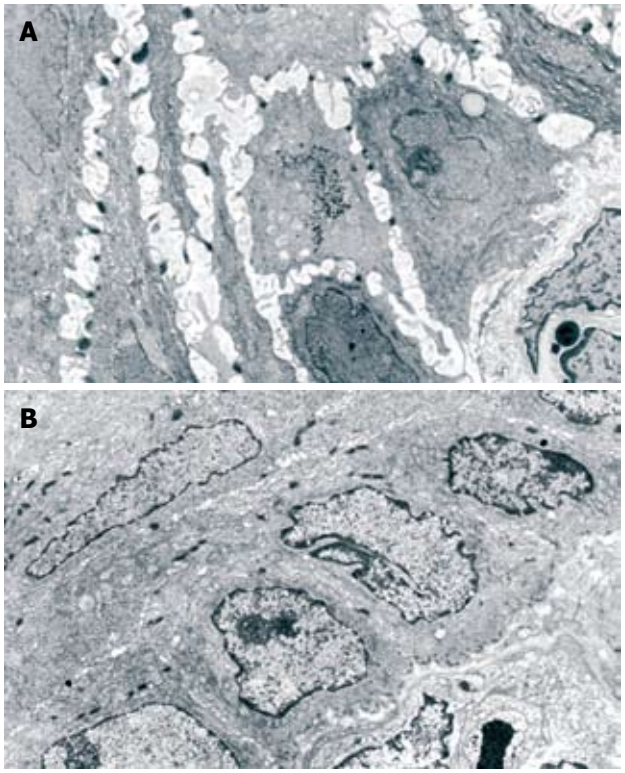

Figure 2 Photomicrographs of esophageal mucosa, obtained using TEM of the suprabasal layer (original magnification, x 3500), showing DIS before (A) and after pantoprazole treatment (B).

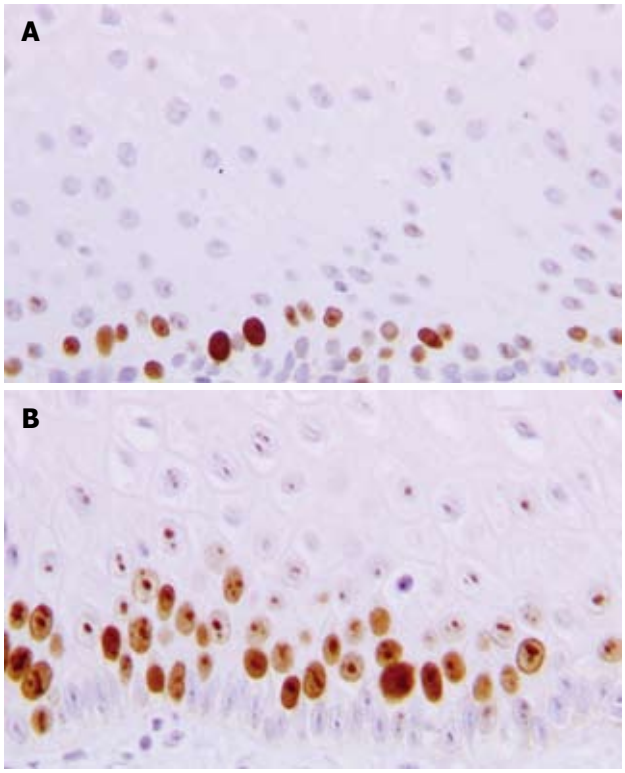

Figure 4 MIB 1 immunostaining of histological sections from an ERD patient at baseline (A) and after 3 mo of therapy (B). Note the increase in the number of proliferating cells after pantoprazole treatment.

their architectural distribution towards the mucosa.

After 3 mo of therapy (T3), the mean MIB1-LI values of NERD and ERD were 37.1% ± 13.2% (range: 22-61.7) and 25.4% ± 10.6% (range: 11-58), respectively (Figure 3). The paired-sample *t*-test, comparing MIB1-LI values measured in each patient at T0 and T3, showed a significant increase in cell proliferation in ERD ($P = 0.006$), but not in NERD patients ($P = 0.78$). In Figure 4, the MIB1-immunostained sections of biopsies taken from the same ERD patient are shown at baseline and after 3 mo of therapy, respectively. A greater number of MIB1-positive cells are clearly visible after therapy.

After 6 mo of therapy (T6), in the 14 ERD and 15 NERD randomized patients, the mean MIB1-LI values of NERD and ERD were 33.3% ± 9.6% and 28.2% ± 5.9%, respectively (Figure 5). Both in NERD and ERD patients, a paired-sample *t*-test for MIB1-LI showed a significant increase in cell proliferation after 6 mo of therapy compared to the baseline ($P < 0.01$), while a significant difference for MIB1-LI was achieved between 3 and 6 mo of therapy only in the ERD group ($P < 0.05$) (Figure 5).

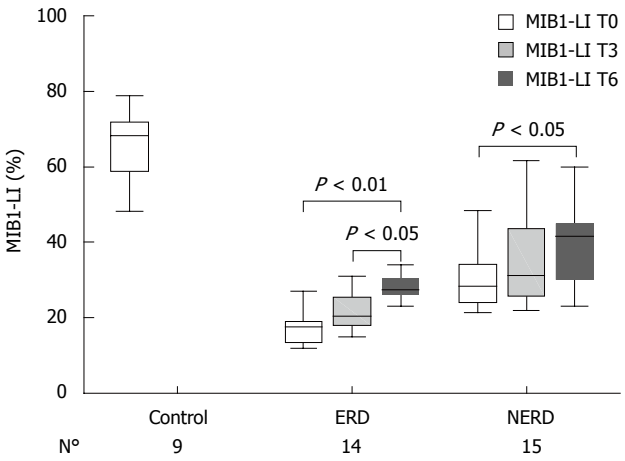

Figure 5 Box plots of MIB1-labelling index (LI), LI median (bold line in the box), and interquartile range (upper and lower lines of the box) in human esophageal mucosa of healthy controls and of randomized patients with ERD and NERD at baseline and after 3 and 6 mo.

DISCUSSION

Evidence is accumulating that erosive endoscopic changes within the esophageal mucosa in patients with GERD accompanied by reflux esophagitis result from a disequilibrium between aggressive factors and protective mechanisms[6-7].

Because aggressive factors always operate on the luminal side of the esophageal mucosa, the esophageal pre-epithelial barrier, represented by a mucus-buffer layer covering the epithelium, plays a role in mucosal protection[6-8].

Recently, we demonstrated that, in patients with GERD, cell proliferation is reduced in esophageal mucosa exposed to chronic acid-peptic insult. In particular, patients with NERD and ERD showed a decrease in cell proliferation to 50% and 75%, respectively, compared to normal subjects[14]. Therefore, esophageal cell proliferation should be taken into consideration as one of the factors involved in the esophageal mucosal defense mechanisms.

In order to better elucidate the relationship between esophageal acid-peptic exposure, ultrastructural alterations of the epithelial lining of the esophagus, and GERD symptoms, our attention was focused on the differences in proliferating activity in NERD and ERD patients.

This study analyzed esophageal-epithelial cell proliferation in patients with gastroesophageal reflux disease with NERD or ERD using immunohistochemical techniques. We investigated whether PPI treatment, reducing the chronic acid-peptic insult, is able to improve esophageal cell proliferation.

After 3 mo, pantoprazole therapy induced, in our subset of patients, ultrastructural healing of mucosal damage in 89% and 93% of ERD and NERD patients, respectively. The ultrastructural healing of the esophageal mucosa was accompanied by a complete resolution of the esophageal symptoms. The patients with an incomplete healing of the DIS after 3 mo of therapy showed persistent symptoms, although these patients became asymptomatic after a further 3 mo of therapy, and showed a complete recovery of the mucosa. These results are similar to our previous study of omeprazole therapy[23].

Our results confirmed that cell proliferation is reduced in esophageal mucosa exposed to chronic acid-peptic insult[14]. In particular, at baseline, the mean MIB1-LI value was 31% and 22% in NERD and ERD patients respectively, compared to 66% in the healthy subjects.

Three months of PPI treatment was able to improve cell proliferation in ERD and NERD patients, though the improvement was only statistically significant in the ERD group. After 6 mo of therapy, we observed a significant further increase in the mean MIB1-LI in both groups of randomized patients. However, even after 6 mo of therapy, the proliferation of the esophageal epithelium in GERD patients did not reach the values of normal subjects.

Two factors regarding the reduced epithelial proliferation activity observed in GERD should be considered. Firstly, the chronic cell damage induced by gastroesophageal reflux could determine a reduction in the proliferation rate of the esophageal epithelium, or a constitutive lower capability for cell proliferation could lead to increased susceptibility to damage induced by gastroesophageal reflux. Our data regarding the cell proliferation rate in the esophageal epithelium after treatment showed that gastroesophageal reflux alone does not induce a decrease in cell proliferation. In fact, we observed that after 3 or 6 mo of PPI treatment, the mean of MIB1-LI in patients with GERD did not reach the mean of that in healthy subjects, although 6 mo of therapy was able to improve the cell proliferation in ERD patients to the same level of NERD patients.

The second factor regards the implied existence of an individual predisposition for the mucosa to react in different ways to acid and pepsin insults. This concept supports the idea that individuals who develop ERD are genetically characterized by a weaker proliferating epithelial cell capability. On the other hand, patients with more efficient epithelial proliferation capacity could have a lower probability of developing macroscopic mucosal lesions when stressed by acid and pepsin. A possible genetic influence in the proliferation capability of the mucosa has a certain appeal.

In conclusion, this is the first demonstration that long-term pantoprazole therapy may induce, in our subset of patients, the ultrastructural healing of mucosal damage both in ERD and in NERD patients. Moreover, long-term pantoprazole treatment could be helpful in increasing the capability for esophageal cell proliferation in GERD, particularly in ERD patients. Further genetic studies are required to better understand the mucosal defense mechanisms and in particular the cellular proliferative activity of esophageal mucosa.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is represented by a broad spectrum of endoscopic and histological features. Esophageal mucosal defense mecha-

nisms, balancing luminal aggressive factors, operate at three overlapping levels: pre-epithelial, epithelial and post-epithelial. The proliferation rate of the esophageal epithelium is inferior in patients affected by GERD compared to normal subjects. Three months of PPI treatment was able to improve cell proliferation in erosive esophagitis (ERD) and NERD patients.

Innovations and breakthroughs

This is the first demonstration that long-term pantoprazole therapy may induce, in our subset of patients, the ultrastructural healing of mucosal damage both in ERD and in NERD patients. Moreover, long-term pantoprazole treatment could be helpful in increasing the capability for esophageal cell proliferation in GERD, particularly in ERD patients.

Applications

Their data are important in the application of therapy in gastroesophageal reflux. It should also be recognized that there are individual differences in the way the mucosa reacts to acid and pepsin insults. This concept supports the idea that individuals who develop ERD are genetically characterized by a weaker proliferating epithelial cell capability. Further studies are needed to evaluate this more fully.

Terminology

GERD indicates chronic symptoms or mucosal damage produced by abnormal reflux in the esophagus. Esophageal mucosa: esophageal mucosa consists of partially keratinized stratified squamous epithelium with three functional regions: stratum corneum, stratum spinosum, and stratum germinativum. Tissue resistance has three protective components: these are designated as preepithelial, epithelial, and postepithelial defenses.

Peer review

In this manuscript, the authors reported ultrastructural healing of esophageal mucosal damage with PPI therapy.

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BRIEF ARTICLES

Angiotensin-receptor blockers as therapy for mild-to-moderate hypertension-associated non-alcoholic steatohepatitis

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Abstract

AIM: To evaluate insulin resistance, cytolysis and non-alcoholic steatohepatitis (NASH) score (NAS) using the Kleiner and Brunt criteria in 54 patients with NASH and mild-to-moderate hypertension, treated with telmisartan *vs* valsartan for 20 mo.

METHODS: All patients met the NCEP-ATP Ⅲ criteria for metabolic syndrome. Histology confirmed steatohepatitis, defined as a NAS greater than five up to 3 wk prior inclusion, using the current criteria. Patients with viral hepatitis, chronic alcohol intake, drug abuse or other significant immune or metabolic hepatic pathology were excluded. Subjects were randomly assigned either to the valsartan (V) group (standard dose 80 mg o.d., *n* = 26), or to the telmisartan (T) group (standard dose 20 mg o.d., *n* = 28). Treatment had to be taken daily at the same hour with no concomitant medication or alcohol consumption allowed. Neither the patient nor the medical staff was aware of treatment group allocation. Paired liver biopsies obtained at inclusion (visit 1) and end of treatment (EOT) were assessed by a single blinded pathologist, not aware of patient or treatment group. Blood pressure, BMI, ALT, AST, HOMA-IR, plasma triglycerides (TG) and total cholesterol (TC) were evaluated at inclusion and every 4 mo until EOT (visit 6).

RESULTS: At EOT we noticed a significant decrease in ALT levels *vs* inclusion in all patients and this decrease did not differ significantly in group T *vs* group V. HOMA-IR significantly decreased at EOT *vs* inclusion in all patients but in group T, the mean HOMA-IR decrease per month was higher than in group V. NAS significantly diminished at EOT in all patients with a higher decrease in group T *vs* group V.

CONCLUSION: Angiotensin receptor blockers seem to be efficient in hypertension-associated NASH. Telmisartan showed a higher efficacy regarding insulin resistance and histology, perhaps because of its specific PPAR-gamma ligand effect.

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Key words: Telmisartan; Valsartan; Non-alcoholic steatohepatitis; Hypertension; Insulin-resistance; Hepatic steatosis; Necroinflammation

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a condition pathogenically linked to the metabolic syndrome by the intervention of insulin resistance (IR), characterized by hepatic steatosis in the absence of significant alcohol use, hepatotoxic medications or other known liver disease[1]. Currently, NAFLD and non-alcoholic steatohepatitis (NASH) are well-recognized causes of progressive chronic liver disease leading to cirrhosis and hepatocellular carcinoma[2-5]. All theories present NAFLD/NASH as the hepatic component of the metabolic syndrome (MS), whose central features include obesity, peripheral insulin resistance, diabetes, dislipidemia, and hypertension[6-8]. Potential therapies tested in NASH treat only the consequences of this condition or try to eliminate excessive fat and target the IR. Reducing food intake can limit accumulation of liver fat and can reverse IR, but there are no well-controlled trials for weight control as a therapy for NAFLD[9]. Other therapeutic interventions, pointing on other features of MS like dislipidemia and impaired glucose tolerance, trying to promote hepatic cytoprotection, or reduction of fibrosis were also evaluated.

This article focuses on angiotensin receptor blockers (ARB's) as multivalent therapeutic agents for NASH, targeting not only hypertension, but also the mechanisms of IR and of hepatic injury *via* renin-angiotensin system (RAS) as prominent pathways of liver damage. The primary endpoints of the study were to prove that ARB's can improve IR in mild-to-moderate hypertensive patients with histologically confirmed NASH, and that monotherapy with ARBs, on regularly basis, can ameliorate cytolysis, while biochemical improvement in these patients correlates with amelioration of NASH activity score. The secondary endpoint was to prove certain superiority of telmisartan *vs* valsartan in NASH-hypertensive patients regarding IR, cytolysis, and necroinflammation, given its specific PPAR-γ modulatory effects.

MATERIALS AND METHODS

Study population and screening

The study conducted between May, 2006 and November, 2007 at Filantropia University Hospital from Craiova-Romania was in accordance with the Helsinki Declaration of 1975, and approved by the Review Ethics Board of the University Medicine and Pharmacy of Craiova and of the Filantropia University Hospital. We screened for MS in 294 patients, using the definition accepted in the 2001 guidelines by the National Cholesterol Education Project Adult Treatment Panel (NCEP-ATP Ⅲ)[10]. Only 159 of 294 patients (54.1%) met the NCEP-ATP Ⅲ criteria (P = 0.179, χ^2 = 1.79) and only 89 of the 159 subjects had mild-to-moderate hypertension (57.8%, P = 0.153, χ^2 = 2.03).

Patients had to give full informed consent, including paired liver biopsies, and to be between 18 and 65 years old. The inclusion criteria also included confirmation of MS by NCE-ATP Ⅲ criteria, in subjects with mild-to-moderate hypertension documented by Holter evaluation up to 4 wk prior inclusion, with systolic BP between 180 mmHg and 135 mmHg and the diastolic between 120 mmHg and 85 mmHg and having ALT values more than 1.5-fold normal range (30 IU/dL maximum normality) for at least at 2 determinations, up to 4 weeks prior inclusion. All patients had to have a fasting plasma glucose (FPG) level less than 130 mg/dL, without any therapy or low-carbohydrate diet for at least three determinations up to two weeks prior inclusion, and histologically[11] confirmed NASH with a necroinflammatory score of 5 or more up to 3 wk before inclusion (according to the scoring system proposed and validated for use in clinical trials by the Pathology Committee of the NASH Clinical Research Network in 2005; available at http://tpis.upmc.com/TPIShome). Other inclusion criteria included acceptance of alcohol abstinence, acceptance of taking the study medication daily at the same hour, and answering at each visit an alcohol consumption questionnaire for monitoring alcohol intake, adapted from Behavioral Risk Factor Surveillance System[12] 2007 Questionnaire (available at http://www.cdc.gov/brfss/questionnaires/english.html). Little amounts of alcohol were allowed occasionally, but not more than two drinks/week (one standard US alcoholic drinks = 14 g pure alcohol). No dietary restrictions or lifestyle modifications were imposed in any case, except current recommendations made by the general practitioner at the regular visits, and no concomitant medication was allowed 1 mo before and after treatment as well as for the entire period of study.

Uncontrolled hypertension or requiring more than a single drug to obtain BP control, history of or confirmed viral hepatitis at screening, drug or alcohol abuse and any other concomitant/pre-existing metabolic or immune hepatic disease were exclusion criteria, as well as normal ALT, HIV positivity and use of dietary supplements, or any other concomitant medication taken on a regularly basis. Dramatic lifestyle changes (e.g. low-calorie diets, intensive physical training, surgery for obesity) were not permitted during the study and patients were encouraged to keep their regular dietary habits and to avoid weight variations. Patients unable to give informed consent, refusing paired liver biopsies, or having any other severe associated organic or psychiatric pathology, neoplasia, or history of intolerance to ARBs were also excluded.

By checking the inclusion/exclusion criteria, only 72/89 patients continued the screening and underwent liver biopsy. The study design scheduled two liver biopsies: the first biopsy performed up to 3 wk prior inclusion (considered as the index biopsy) and the second biopsy obtained at maximum 2 wk after the end of treatment. A single pathologist, unaware of patient information, evaluated the histological features of both index and second biopsies. NAFLD activity scores (NAS) were assessed in each case, and patients with simple steatosis, or those not fully meeting all criteria for steatohepatitis (18/72), were excluded. We finally included 54 subjects (28M/26F) in the trial.

Subjects were randomly assigned using dedicated computer software either to the valsartan (V) group (receiving a standard dose of 80 mg o.d., n = 26), or to the telmisartan (T) group (standard dose 20 mg o.d., n = 28). Medication was blinded and treatment had to be taken daily at the same hour in the morning, with no concomi-

tant medication or alcohol consumption allowed. Neither the patient nor the medical staff was aware of the treatment group allocation.

Biochemical analyses and histology

A central laboratory used standard procedures to insure reproducibility. FPG, alanine-aminotransferase (ALT), aspartate-aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), bilirubin (B), total cholesterol (TC), and triglycerides (TG), were determined on fresh serum using an autoanalyzer Hitachi 917 Automate with Roche Diagnostics reagents. Serum samples obtained after an overnight fast of at least 12 h and immediately frozen at -20℃ were used to determine the levels of immunoreactive insulin (IRI) by a chemiluminescence immunoassay (Elecsys Modular Analytics E170; Roche Diagnostics) using monoclonal antibodies with stated negligible cross-reactivity. We determined IR by the homeostasis model assessment (HOMA-IR) method[13] using the following equation: HOMA-IR = [FPG (mg/dL) × IRI (μU/mL)]/405.

The percutaneous liver biopsy technique was performed in all cases[14]. All biopsies were fixed, paraffin-embedded, and stained with hematoxylin-eosin and Masson's trichrome/picrosirius red for collagen. Biopsies were evaluated by a single, experienced, blinded pathologist, not aware about allocation in one or another treatment group and about the clinical and biochemical parameters of any patient using the scoring system validated by Kleiner *et al*[11]. As known, this histology scoring system quantifies necroinflammatory and steatotic changes (steatosis, lobular inflammation, and ballooning) resulting NAFLD activity scores (NAS) that range between 0 and 8. Scores greater or equal to 5 are largely diagnostic for NASH, while scores less than 4 characterize a fatty liver having simple steatosis, but not NASH. Fibrotic changes are evaluated separately from NAS, ranging from 0 (no fibrosis) to 4 (cirrhosis). Our study also assessed the fibrosis stage in all patients in order to evaluate the antifibrotic effects of the two ARBs.

Study schedule and surveillance parameters

After screening, the included patients were followed for 20 mo. The study flowchart previewed 6 visits (V1-V6) scheduled every 4 mo (112 d) with a ± 5 d deviation admitted. Each visit took place between 8.00 and 11.00 a.m. and consisted with a clinical examination, blood pressure (BP) and body mass index (BMI) determinations, serum sampling, and a questionnaire. An average of three successive determinations of systolic (sBP) and diastolic (dBP) BP was calculated and used in records each visit. BMI was computed using the formula: [weight (kg)]/[square of height (meters)], while serum was collected for FPG, ALT, AST, GGT, B, TC, TG and IRI determinations. An alcohol consumption questionnaire was also administered each visit and study compliance was strictly monitored, including checking the returned medication. Additionally, V1 (inclusion) comprised recording of the result of the index liver biopsy which was performed -21 to -7 d previously, while V6 ended with the second liver biopsy, performed at maximum 2 wk after the end-of treatment (EOT).

The primary parameters at followed-up were s/dBP, BMI, ALT, AST, GGT, HOMA-IR, TC, TG, NAS and fibrosis scores. Additionally, we used in the analysis the following derivate parameters: the mean monthly decreases of ALT (mMd*ALT), HOMA-IR (mMd*HOMA-IR), TC (mMd*TC) and TG (mMd*TG), the mean decrease for NAS (md*NAS) and fibrosis score (md*Fibrosis) and the mean decrease of s/dBP (md*s/dBP) and BMI (md*BMI). The mMd*ALT, mMd*HOMA-IR, mMd*TC and mMd*TG represent the difference between the average values of respectively, ALT, HOMA-IR, TC and TG, between V2 and V6 and their mean values at V1 divided by the number of months of follow-up (20 mo for the patients that fully completed treatment), mathematically expressed by the following formula (where *"y"* is the study parameter, *"i"* is the number of the visit, *"N_i"* is the number of months of follow-up and *"V_i"* is the index of the visit):

$$mMd * y = \left[\frac{\sum_{i=2}^{i} \frac{\overline{y(V_i)}}{i-1} - \overline{y(V_1)}}{N_i}\right]$$

The md*NAS and md*Fibrosis were calculated by subtracting the average NAS and, respectively, fibrosis scores at index biopsy from those recorded at V6, while the md*s/dBP and md*BMI represent the difference between the respective values of these parameters averaged from V2 to the last visit and their mean value at V1 without considering the number of months of follow-up, as in the subsequent formula (where *"z"* is the study parameter, *"i"* is the number of the visit, and *"V_i"*

$$mMd * z = \left[\frac{\sum_{i=2}^{i} \frac{\overline{z(V_i)}}{i-1} - \overline{z(V_1)}}{}\right]$$

Statistical analysis

Data is presented as mean ± SE. Differences in the baseline parameters between groups T and V were tested by the Kruskal-Wallis test to check for any baseline bias. Normal distribution was tested using the Kolmogorov-Smirnov test while the Wilcoxon test was used to assess the differences between the paired observations. Other data recorded during the study from groups T and V were analyzed by one-way analysis of variance ANOVA. A statistically significant result was considered when P value was less than 0.05. All statistical analyses were performed using the MedCalc Software Version 10.0.2.0-2008 (MedCalc Software, Broekstraat 52, 9030 Mariakerke, Belgium).

RESULTS

Mean age for the included patients was 48.89 ± 1.41 (48 ± 1.98 in group T and 49.85 ± 2.05 in group V) while the average dose per BMI unit was 0.74 ± 0.01 mg telmisartan in group T and 2.92 ± 0.06 mg valsartan in group V. No statistically significant difference between the two groups regarding the demographic data, as well as among the survey parameters, existed at inclusion.

Table 1 Demographics and baseline data at inclusion (mean ± SE)

	Units	Overall patients	Group		*P*
			T	V	
No. of patients		54	28	26	NS
Gender	Male/female	28/26	14/14	12/14	NS
Age	yr	48.89 ± 1.41	48 ± 1.98	49.85 ± 2.05	
BMI (V1)	kg/m^2	27.42 ± 0.36	27.21 ± 0.51	27.65 ± 0.53	NS
dBP (V1)	mmHg	101.65 ± 1.05	101.43 ± 1.17	101.89 ± 1.8	
sBP (V1)	mmHg	156.53 ± 1.28	155.71 ± 1.61	157.42 ± 2.04	
ALT (V1)	IU/L	67.65 ± 2.01	66.96 ± 2.95	68.38 ± 2.75	NS
AST (V1)	IU/L	70.31 ± 2.34	72.78 ± 3.53	67.65 ± 3.02	
GGT (V1)	IU/L	23.85 ± 1.62	22.60 ± 2.37	25.19 ± 2.22	
B (V1)	mg/dL	0.89 ± 0.02	0.9 ± 0.03	0.88 ± 0.04	
HOMA IR (V1)	units	7.7 ± 0.24	7.81 ± 0.40	7.58 ± 0.25	
IRI (V1)	μU/mL	27.64 ± 0.86	27.78 ± 1.14	27.50 ± 1.32	
FPG (V1)	mg/dL	113.83 ± 1.98	113.60 ± 2.77	114.07 ± 2.88	
HDL-C (V1)	mg/dL	42.44 ± 1.2	42.34 ± 1.68	42.54 ± 1.77	NS[1]
		M: 37.8 ± 1.38	M: 37.45 ± 1.86	M: 38.16 ± 2.11	
		F: 47.42 ± 1.5	F: 47.23 ± 2.13	F: 47.66 ± 2.19	
TC (V1)	mg/dL	196.46 ± 1.13	196.07 ± 1.5	196.88 ± 1.73	NS[1]
		M: 200.82 ± 1.2	M: 200.57 ± 1.29	M: 201.07 ± 2.07	
		F: 191.77 ± 1.5	F: 191.57 ± 2.13	F: 192 ± 2.19	
TG (V1)	mg/dL	161.51 ± 4.91	165.64 ± 6.95	157.08 ± 7.18	
NAS (V1)	points	5.89 ± 0.14	6 ± 0.18	5.77 ± 0.22	NS
Steatosis (V1)		2.16 ± 0.09	2.21 ± 0.12	2.11 ± 0.16	
Lobular inflammation (V1)		1.70 ± 0.06	1.68 ± 0.09	1.73 ± 0.09	
Ballooning (V1)		2.02 ± 0.1	2.11 ± 0.13	1.92 ± 0.16	
Fibrosis (V1)	points	2.11 ± 0.11	2.07 ± 0.16	2.15 ± 0.15	NS

BMI: Body mass index; dBP: Diastolic blood pressure; sBP: Systolic blood pressure; ALT: Alanine-aminotransferase; AST: Aspartate-aminotransferase; GGT: Gamma-glutamyl transpeptidase; B: Bilirubin; HOMA-IR: Homeostasis model assessment index for insulin-resistance; IRI: Plasma immunoreactive insulin; FPG: Fasting plasma glucose; TC: Total cholesterol; HDL-C: High density lypoprotein-cholesterol; TG: Triglycerides; NAS: NASH activity score; M: Male patients; F: female patients; V1: index data at visit 1; [1]Statistics performed depending on gender and also in overall patients.

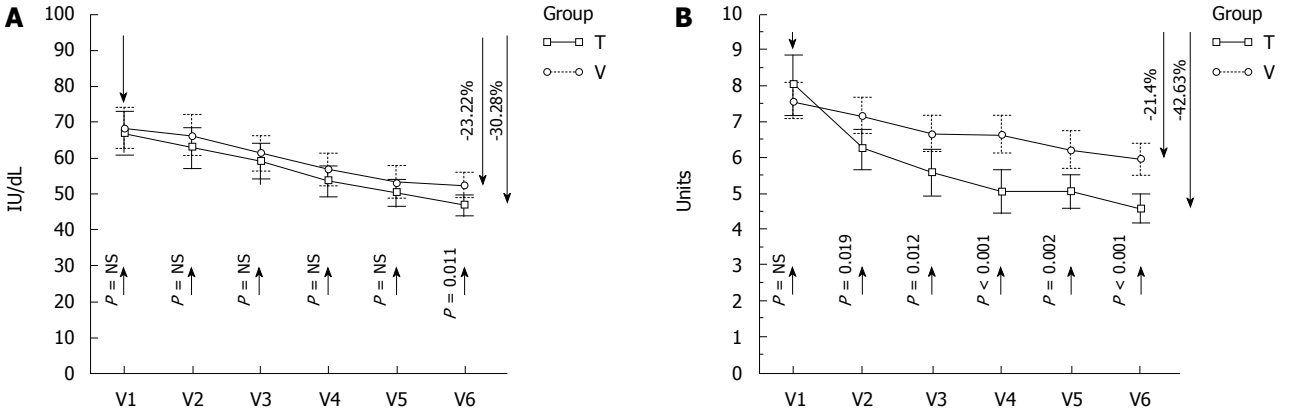

Figure 1 Primary parameters of the biochemical study. Comparative dynamics for ALT and HOMA-IR from V1 to V6 in the study groups. A: ALT variation from V1 to V6; B: HOMA-IR variation from V1 to V6. ALT: Alanine-aminotransferase; HOMA-IR: Homeostasis model assessment index for insulin-resistance; V1 to V6: Number of scheduled visit; T: Telmisartan study group; V: Valsartan study group; NS: Not statistically significant.

Table 1 shows a synopsis of all the survey parameters at baseline in all included patients, as well as in the two therapeutic groups.

All included patients finished the study. At the end of the study, we observed significant differences regarding biochemical, metabolic, histological and hemodynamic parameters in both study groups compared with inclusion data. Tables 2 and 3 review the main results of the study concerning both the primary parameters of survey as well as the derivate ones.

Cytolysis study

ALT values at V6 were significantly lower versus inclusion in all patients (49.48 ± 1.16 IU/L *vs* 67.65 ± 2.01 IU/L, $P < 0.001$), although the values did not returned to normality in either group. Both therapeutic groups had significantly lower ALT levels at EOT compared to V1; however, in group T these values were significantly smaller than in group V (46.68 ± 1.42 IU/L *vs* 52.50 ± 1.70 IU/L, $P = 0.011$). Despite a constant decrease of ALT in both groups from V2 to

Table 2 Comparative overview of the primary parameters at inclusion versus end-of-treatment in study groups and in overall patients

	Units	Overall		Group T		Group V	
		mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*
sBP (V1)	mmHg	156.54 ± 1.28	< 0.0001	155.71 ± 1.61	< 0.0001	157.42 ± 2.04	< 0.0001
sBP (V6)		134.24 ± 1.28		133.21 ± 1.23		135.35 ± 2.31	
dBP (V1)	mmHg	101.65 ± 1.05	< 0.0001	101.43 ± 1.17	< 0.0001	101.89 ± 1.8	< 0.0001
dBP (V6)		77.8 ± 1.04		77.14 ± 1.25		78.5 ± 1.7	
ALT (V1)	IU/L	67.65 ± 2.01	< 0.001	66.96 ± 2.95	< 0.001	68.38 ± 2.75	< 0.001
ALT (V6)		49.48 ± 1.16		46.68 ± 1.42		52.50 ± 1.70	
AST (V1)	IU/L	70.31 ± 2.34	< 0.001	72.78 ± 3.53	< 0.001	67.65 ± 3.02	< 0.001
AST (V6)		51.11 ± 1.83		47.57 ± 2.08		54.92 ± 2.95	
GGT (V1)	IU/L	23.85 ± 1.62	NS	22.60 ± 2.37	NS	25.19 ± 2.22	NS
GGT (V6)		21.02 ± 1.98		21.96 ± 3.55		20 ± 1.58	
B (V1)	mg/dL	0.89 ± 0.02	NS	0.9 ± 0.03	NS	0.88 ± 0.04	NS
B (V6)		0.94 ± 0.02		0.94 ± 0.03		0.93 ± 0.03	
BMI (V1)	kg/m^2	27.42 ± 0.36	NS	27.21 ± 0.51	NS	27.65 ± 0.53	NS
BMI (V6)		26.96 ± 0.36		26.93 ± 0.49		27.00 ± 0.54	
HOMA-IR (V1)	mg × μIU	7.7 ± 0.24	< 0.001	7.81 ± 0.40	< 0.001	7.57 ± 0.25	< 0.05
HOMA-IR (V6)	/dL × 10^{-2}	5.19 ± 0.18		4.48 ± 0.21		5.95 ± 0.22	
TG (V1)	mg/dL	161.51 ± 4.98	< 0.05[1]	165.64 ± 6.95	< 0.031[1]	157.08 ± 7.18	NS[1]
		F: 168.42 ± 8.28		F: 169.85 ± 11.2		F: 166.75 ± 12.82	
		M: 155.10 ± 5.63		M:161.42 ± 8.49		M: 148.78 ± 7.31	
TG (V6)		153.96 ± 4.8		154.14 ± 6.79		153.77 ± 6.93	
		F: 160 ± 8.29		F: 156.78 ± 11.33		F: 163.75 ± 12.61	
		M: 148.35 ± 5.09		M: 151.5 ± 7.88		M: 145.21 ± 6.65	
TC (V1)	mg/dL	196.46 ± 1.13	< 0.008[1]	196.07 ± 1.5	< 0.024[1]	196.88 ± 1.73	NS[1]
		F: 191.77 ± 1.5		M: 200.57 ± 1.29		M: 201.07 ± 2.07	
		M: 200.82 ± 1.2		F: 191.57 ± 2.13		F: 192 ± 2.19	
TC (V6)		194.04 ± 1.19		191.89 ± 1.64		196.35 ± 1.64	
		F: 189.73 ± 1.58		F: 188 ± 2.23		F: 191.75 ± 2.19	
		M: 198.04 ± 1.39		M: 195.79 ± 1.96		M: 200.29 ± 1.87	
NAS (V1)	Point	5.89 ± 0.14	< 0.001[1]	6 ± 0.18	< 0.045[1]	5.77 ± 0.22	NS[1]
Steatosis		2.16 ± 0.09	[2]Excepting inflammation (V1) *vs* (V6)	2.21 ± 0.12		2.11 ± 0.16	[2]Excepting steatosis (V1) *vs* (V6) *P* = 0.027
Inflammation		1.70 ± 0.06	NS	1.68 ± 0.09		1.73 ± 0.09	
Balooning		2.02 ± 0.1		2.11 ± 0.13		1.92 ± 0.16	
NAS (V6)		4.96 ± 0.14		4.57 ± 0.18		5.38 ± 0.2	
Steatosis		1.74 ± 0.09		1.71 ± 0.11		1.77 ± 0.15	
Inflammation		1.55 ± 0.10		1.32 ± 0.12		1.81 ± 0.14	
Balooning		1.67 ± 0.08		1.53 ± 0.11		1.81 ± 0.13	
Fibrosis (V1)	Point	2.11 ± 0.11	< 0.001	2.07 ± 0.16	< 0.001	2.15 ± 0.15	NS
Fibrosis (V6)		1.57 ± 0.09		1.32 ± 0.13		1.84 ± 0.11	

sBP: Systolic blood pressure; dBP: Diastolic blood pressure; ALT: Alanine-aminotransferase; AST: Aspartate-aminotransferase; GGT: Gamma-glutamyl transpeptidase; B: Bilirubin; BMI: Body mass index; HOMA-IR: Homeostasis model assessment index for insulin-resistance; TG: Triglycerides; TC: Total cholesterol; NAS: NASH activity score; M: Male patients; F: Female patients; V1: Index data at visit 1; V6: End-of-treatment data; [1]For any comparation; [2]Exception.

V6 with differences in favor of group T (Figure 1A), significantly lower values in this group, as compared to group V, were observed only at the last visit. As Table 2 shows, similar data with significantly lower values in group T *vs* V (47.57 ± 2.08 *vs* 52.50 ± 1.70, $P = 0.044$) were noticed for AST, but not for GGT and B which remained stable in both groups throughout the study.

The overall mMd*ALT value was -0.57 ± 0.05 IU/L per month, with -0.63 ± 0.09 IU/L/mo in group T and -0.52 ± 0.05 in group V (Figure 2A). No significant difference between groups regarding this aspect was observed either.

Metabolic study

BMI was stable during the study in all patients (27.42 ± 0.36 *vs* 26.96 ± 0.36, *P* = NS) with no difference between group T and V at V6 (26.93 ± 0.49 in group T *vs* 27.00 ± 0.54, *P* = NS) and no differences were found between the two groups regarding the md*BMI (0.11 ± 0.47 in group T *vs* -0.05 ± 0.57 in group V, *P* = NS). At EOT, HOMA-IR was 5.19 ± 0.18, significantly lower than 7.7 ± 0.24 as found at V1 in overall patients ($P < 0.001$). Although this parameter significantly decreased in both groups compared to inclusion demonstrating an amelioration of insulin-sensitivity by both ARBs ($P < 0.01$), in group T this improvement was more important than in group V with $P < 0.001$ when comparing the HOMA-IR (V6) values between group T and V (4.48 ± 0.21 *vs* 5.95 ± 0.22). As shown in Figure 1B, the HOMA-IR constantly decreased in both groups, with significantly lower values for group T *vs* V from V2 to V6, proving a better insulin-sensitizing activity for telmisartan. Moreover, the mMd*HOMA-IR,

Table 3 Derivate study parameters in treatment groups and in overall patients (mean ± SE)

	Units	Overall	Group T	Group V
mMd*ALT	IU/dL per month	-0.57 ± 0.05	-0.63 ± 0.09	-0.52 ± 0.05
mMd*HOMA-IR	units/mo	$-9.63 \pm 0.94 \times 10^{-2}$	$-13.7 \pm 1.32 \times 10^{-2}$	$-5.3 \pm 0.64 \times 10^{-2}$
mMd*TG	mg/dL per month	-1.79 ± 0.10	-1.99 ± 0.16	-1.58 ± 0.11
mMd*TC	mg/dL per month	-0.03 ± 0.03	-0.12 ± 0.05	0.06 ± 0.02
md*BMI	units	0.03 ± 0.36	0.11 ± 0.47	-0.05 ± 0.57
md*sBP	mmHg	-21.13 ± 1.13	-21.35 ± 1.68	-20.90 ± 1.54
md*dBP	mmHg	-19.18 ± 1.43	-19.65 ± 2.15	-18.67 ± 1.91
md*NAS	point	-0.92 ± 0.14	-1.43 ± 0.19	-0.38 ± 0.17
md*Fibrosis	point	-0.46 ± 0.11	-0.75 ± 0.13	-0.15 ± 0.18

mMd*ALT: Mean monthly decrease of ALT; mMd*HOMA-IR: Mean monthly decreases for HOMA-IR; mMd*TG: Mean monthly decrease of plasma triglycerides; mMd*TC: Mean monthly decrease of total cholesterol; md*BMI: Mean decrease of BMI; md*sBP: Mean decrease of systolic blood pressure; md*dBP: Mean decrease of diastolic blood pressure; md*NAS: The mean decrease for NAS; md*Fibrosis: Mean decrease for fibrosis score.

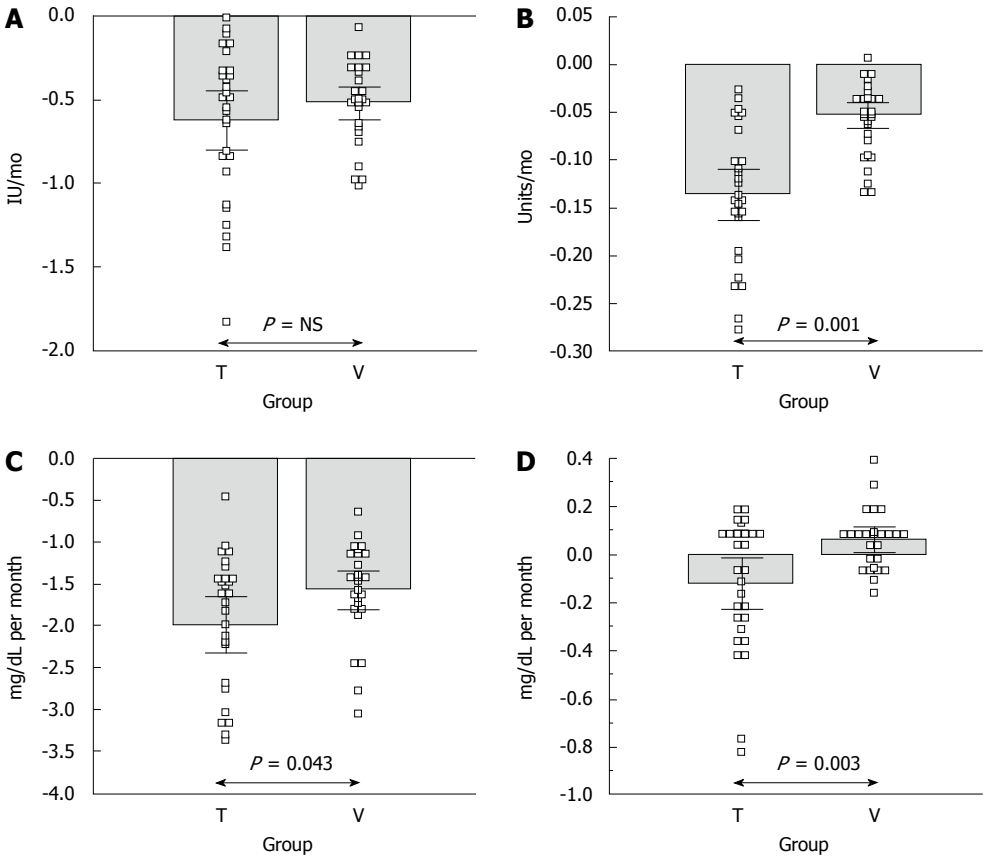

Figure 2 Derivate parameters of the biochemical study. Comparisons (box-and whisker means) of the averaged decreases per month for ALT, HOMA-IR, TC and TG in the two study groups. A: Mean monthly decrease for ALT; B: Mean monthly decrease of HOMA-IR; C: Mean monthly decrease of triglycerides; D:Mean monthly decrease of total cholesterol. ALT: alanine-aminotransferase; HOMA-IR: homeostasis model assessment index for insulin-resistance; TG: triglycerides; TC: total cholesterol; T: Telmisartan study group; V: Valsartan study group; NS: Not statistically significant.

which was $-9.63 \pm 0.94 \times 10^{-2}$ units/mo in overall patients, was more than two-fold higher in group T with $-13.7 \pm 1.32 \times 10^{-2}$ *vs* $-5.3 \pm 0.64 \times 10^{-2}$ units/mo in group V ($P = 0.001$), demonstrating a reliable effect of telmisartan to improve insulin resistance (Figure 2B).

Lipid profiles were also modified at the EOT. We noticed a decrease of TG values in patients at V6 compared to V1 (153.96 ± 4.8 mg/dL *vs* 161.51 ± 4.98 mg/dL, $P = 0.003$) in both in male and female patients, as shown in Table 2. However, only in group T was the decrease of plasma TG found to be statistically significant by the Wilcoxon test for paired samples (154.14 mg/dL ± 6.79 *vs* 165.64 ± 6.95 mg/dL, $P = 0.0013$). Moreover, although the mean values for TG were similar in groups T and V at V6, the mMd*TG was significantly higher in the telmisartan group, with -1.99 ± 0.16 mg/dL per month *vs* -1.58 ± 0.11 in group V ($P = 0.043$), irrespective of gender of patients (Figure 2C). TC decreased at V6 compared to V1 both in men (198.04 ± 1.39 mg/dL *vs* 200.82 ± 1.2 mg/dL, $P = 0.006$) as in women (189.73 ± 1.58 mg/dL *vs* 191.77 ± 1.5 mg/dL, $P = 0.008$) and in overall patients (194.04 ± 1.19 mg/dL *vs* 196.46 ± 1.13 mg/dL, $P = 0.003$). We did not noticed any difference regarding these values at V6 between groups T and V, when analyzing the results either by gender or in overall patients (191.89 ± 1.64 mg/dL *vs* 196.35 ± 1.64 mg/dL, $P = 0.06$). However, at EOT group T had significant lower values compared with inclusion in both

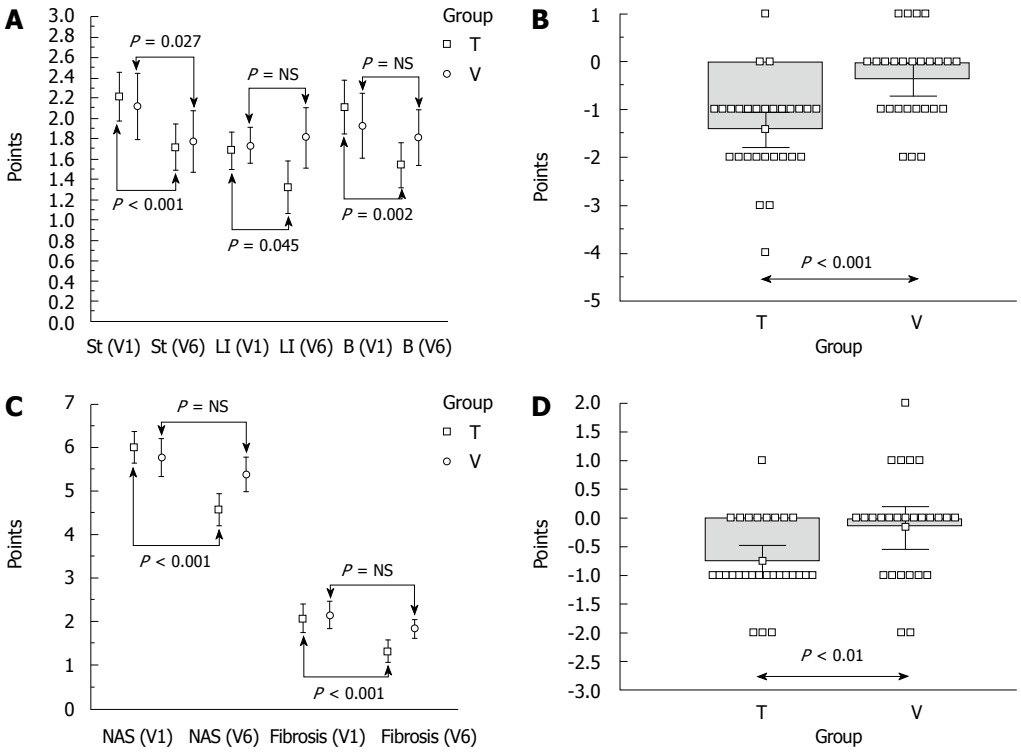

Figure 3 Histology study. Comparisons of averaged decreases for NAS and its components and for the fibrosis scores between V1 and V6 among the study groups. A: Comparison between the values of NAS components at V6 *vs* V1; B: Mean NAS decrease in groups T and V; C: Comparison between the values of NAS and respectively fibrosis scores at V6 *vs* V1; D: Mean decrease of fibrosis scores in groups T and V. St: Steatosis; LI: Lobular inflammation; B: Ballooning; NAS: NASH activity score; V1: Index data at visit 1; V6: End-of-treatment data; NS: Not statistically significant.

male (195.79 ± 1.96 mg/dL *vs* 200.57 ± 1.3 mg/dL, $P = 0.024$) and female patients (188 ± 2.23 mg/dL *vs* 191.57 ± 2.13 mg/dL, $P = 0.006$) while in group V we did not observed the same aspect. Furthermore, as showed in Figure 2D, the mMd*TC was higher in group T than in group V (-0.12 ± 0.05 mg/dL *vs* -0.06 ± 0.02 mg/dL per month, $P = 0.003$) demonstrating a significant effect on the lipid profile by telmisartan, whereas valsartan seemed to lack this property.

Histology study

NAS score decreased at EOT in overall patients (5.89 ± 0.14 *vs* 4.96 ± 0.14, $P < 0.01$), but only steatosis and ballooning showed a significant reduction, while lobular inflammation rested unchanged. The NAS score at V6 was lower in group T *vs* V (4.57 ± 0.18 *vs* 5.38 ± 0.2, $P = 0.004$) demonstrating a significant efficacy of telmisartan to improve hepatic histology. Additionally, when comparing the evolution of the NAS elements in the two groups, we found that all these components significantly decreased in group T from V1 to V6 ($P < 0.045$ for any comparison V1 *vs* V6 concerning steatosis, lobular inflammation and ballooning), while in group V, only steatosis improved ($P = 0.027$) without significant changes for inflammation and ballooning (Figure 3A). Furthermore, the md*NAS was significantly higher in telmisartan group (-1.43 ± 0.19 *vs* -0.38 ± 0.17, $P < 0.001$) confirming that this ARB can effectively act as a factor promoting amelioration of the NASH activity score (Figure 3B).

In all groups, the fibrosis scores at V6 were lower than those observed at V1 (1.57 ± 0.09 *vs* 2.11 ± 0.11, $P < 0.001$); however, fibrosis scores at EOT were higher in group V than in group T (1.84 ± 0.11 *vs* 1.32 ± 0.13, $P = 0.013$). The decrease of the fibrosis score from V1 to V6 was statistically significant in group T (2.07 ± 0.16 to 1.32 ± 0.13, $P < 0.001$), but not in group V, demonstrating an antifibrotic effect of telmisartan that is not possessed by the other ARB (Figure 3C). Moreover, the md*Fibrosis was significantly higher in group T than in group V (-0.75 ± 0.13 *vs* -0.15 ± 0.18, $P = 0.01$), confirming the capacity of telmisartan to inhibit liver fibrosis (Figure 3D).

Hemodynamic study

A detailed analysis of the antihypertensive effect of the two ARBs was not the scope of this study. We only noticed that both drugs are equally potent in reducing both sBP and dBP. Telmisartan reduced BP from 155.71 ± 1.61/101.43 ± 1.17 mmHg at V1 to 133.21 ± 1.23/77.14 ± 1.25 mmHg at V6, while valsartan reduced BP from 157.42 ± 2.04/101.89 ± 1.8 at V1 to 135.35 ± 2.31/78.5 ± 1.7 at V6. No differences were noticed between groups regarding either sBP or dBP at any of the visits from V2 to V6, while the md*sBP and md*dBP values were, respectively, -21.35 ± 1.68 mmHg in group T *vs* -20.90 ± 1.54 in group V and -19.65 ± 2.15 mmHg in group T *vs* -18.67 ± 1.91 in group V (P = NS for both comparisons).

DISCUSSION

In brief, our study demonstrates that although it does not normalize ALT values, telmisartan can reduce cytolysis by 30.28% and can improve IR by decreasing HOMA-IR with 42.63% in patients with NASH and mild-to-moderate hypertension. This improvement is associated with a significant decrease of NAS and fibrosis scores and with an amelioration of the lipid profile demonstrated by lower values of plasma TG and TC in both men and women. On the other hand, despite a significant reduction of ALT levels by 23.22%

and of HOMA-IR by 21.4%, valsartan did not improve liver histology (except steatosis) and had no effect on plasma lipids. There is no statistically significant difference in ALT reduction between the two ARBs, but the higher rates of HOMA-IR reduction, as well as the improvement of NAS score and antifibrotic effect observed in group T, suggests that the effects of this ARB are driven not only through the angiotensin-1 receptor blockade, but also *via* its PPAR-γ modulator specific effects.

Telmisartan is an ARB possessing unique qualities of PPAR-γ modulation that makes it ideal for the treatment of NASH. Unfortunately, no major studies have been performed to confirm its efficacy in steatohepatitis, although a theoretical and experimental fundament exists[15]. Interestingly, a study by Fujita *et al*[16] tested the same compounds as we did in this study in a rat model of NASH, providing evidence that telmisartan, but not valsartan, improved both qualitatively and quantitatively hepatic steatosis, inflammation, and fibrosis. Furthermore, in both rats with choline-deficient diet-induced NASH (*in vivo*) and in primary hepatic stellate cells (*in vitro*), Jin *et al*[17] concluded that telmisartan is able to prevent liver fibrosis by increasing matrix-metaloproteinase (MMP) expression, down-regulation of transforming growth factor beta-1 (TGF-β1) and tissue inhibitor of matrix-metalloproteinases (TIMP), and by inhibition of hepatic stellate cell (HSC) activation and proliferation. A study by Sugimoto *et al*[18] provides evidence that in hepatic steatosis telmisartan (and not valsartan) reduces accumulation of visceral fat and hepatic triglyceride levels, decreases adipocyte size, and increases the muscle expression of certain important genes involved with energy metabolism. These properties of telmisartan are probably linked to its ability to modulate PPARγ activity. Indeed, in a recent study, Yoshida *et al*[19] demonstrated that telmisartan improves IR in advanced glycation end-product (AGE)-exposed human hepatoma (Hep3B) cells by decreasing serine phosphorylation and enhancing tyrosine phosphorylation of insulin-receptor substrate-1 but, when antagonized with an inhibitor of PPARγ, it loses these properties. Other animal studies[20-23] provided additional evidence of properties of telmisartan linking it to PPAR modulation that can account for its effects in steatohepatitis, for example a partial PPAR-α agonist activity which seems to be restricted to the liver, regulating serum adipokines with increased adiponectin and decreased resistin levels, and even anti-inflammatory properties.

Human studies employing ARBs in NASH are quite rare[24-26], testing habitually losartan and lacking either a sufficient number of patients, either an adequate assessment of morphologic changes given the difficulty to obtain paired liver biopsies. The major strength of our study is that, from our knowledge, it is the first human blinded trial evaluating the effects of telmisartan and valsartan in steatohepatitis that uses paired liver biopsies simultaneously with cytolysis and IR assessment. Interestingly, although not pointing on steatohepatitis, a recent study by Ichikawa[27] demonstrated that in hypertensive patients with MS, receiving 20 mg telmisartan daily for 4 wk, resulted in a reduction of HOMA-R by 16%, while 40 mg valsartan/day did not show significant results on this parameter. There are differences between this study and our trial, including different dosage for valsartan (higher doses in our study), longer period of survey (20 mo *vs* 4 mo), younger study population (49 years *vs* 65 years), higher values of HOMA-IR (7.7 units *vs* 3 units), use of Japanese criteria for MS and permission for concomitant medication, but in all, our results confirm the insulin-sensitizing effect of telmisartan. Additionally, we demonstrated that this ARB has a favorable effect on plasma TG and TC in opposition to Ichikawa and other groups[28,29], but in accordance with others[30-34]. As for valsartan, again in contrast with Ichikawa, but in accordance with larger studies[35,36], we demonstrated that it also reduces IR, although it has no other effects on lipid profiles.

There are interesting theories and experimental facts that can explain the intervention of the RAS in liver disease, leading to the theoretical conclusion that ARBs have the capacity to become the first-class option for a tailored therapy in NAFLD and NASH. The RAS is an enzymatic cascade in which renin, an aspartic protease released from juxtaglomerular cells, cleaves angiotensinogen to form a decapeptide, angiotensin Ⅰ (Ang-Ⅰ), which is in turn transformed to angiotensin Ⅱ (Ang-Ⅱ) by the angiotensin-converting enzyme (ACE). Ang-Ⅱ can be further converted by aminopeptidases A and N in Ang-Ⅲ (2-8) which is finally transformed in Ang-Ⅳ (3-8)[37]. Historically, Ang-Ⅱ was first described as the primary effector of this system, but more recent research added new components as a result of the action of prolylendopeptidase and carboxypeptidases: angiotensin 1-5 (Ang-1-5), angiotensin 1-7 (Ang-1-7), and angiotensin 1-9 (Ang-1-9)[38]. Ang-1-7 is a heptapeptide generated from either Ang-Ⅰ or Ang-Ⅱ by a homologue of ACE, angiotensin converting enzyme 2 (ACE2) which has a catalytic domain different from ACE and acts antagonistically as a counter-regulatory factor. The biological actions of Ang-1-7 are both activation of peripheral vasodilatory mechanisms and antitrophic effects mediated by the inhibition of protein synthesis[39,40].

Classically, Ang-Ⅱ and Ang-Ⅲ acts on two types of G-protein-coupled receptors, AT1 and AT2. The AT1 receptor is widely expressed in various tissues (heart, kidney, vessels, liver and adipocytes), while AT2 has low levels of expression after birth, but may play a role in activation of AT1, modulation of cell differentiation, tissue repair and apoptosis[41]. Ang-Ⅳ possesses its own receptors (AT4) distinct from AT1 and 2, and Ang-1-7 acts through a different G protein-coupled receptor (Mas) downregulating AT1[42].

Although consistent convergent data about the intervention of the RAS in NAFLD/NASH exists, the contribution of this factor in setting and promoting the hepatic consequences of MS is still not fully clarified. It is likely that the mechanisms by which RAS could inter-

fere with the pathogenic course that links IR to steatohepatitis might include interactions with insulin receptors and intracellular signalling, effects on adipogenesis, influences on cytokine and adipokine production, interferences with pancreatic β-cell insulin secretion and/or local hepatic effects interfering hepatocellular regulatory mechanisms.

Angiotensinogen is synthesized in the liver and adipocytes, but adipose tissue differs from liver given the differences in the AT1/AT2 receptor populations, the inhibitory effect of the AT2 receptors impairing excessive angiotensinogen production by the adipocytes[43]. RAS is frequently activated in the patients with chronic liver diseases, promoting mainly fibrosis, with Ang-Ⅱ stimulating contractility and proliferation of the activated HSCs, increasing TGF-β1 and promoting neovascularization and production of vascular endothelial growth factor[44]. It is largely accepted[45] that the local hepatic RAS system acts almost exclusively through the AT1 receptors localized to hepatocytes, bile duct epithelial cells, HSCs, myofibroblasts, Kupffer cells and vascular endothelium, as the AT2 receptors are not significantly expressed in liver. However, some data regarding the AT2 receptors exists suggesting that it may have protective effects against fibrosis[46].

Although the profibrotic effects of RAS begins to be unveil in various conditions including NASH, little is known about the inflammatory changes that precede fibrosis. Perfusion studies by Bataller and colleagues showed mild portal inflammation, thickening and thrombosis of small hepatic vessels, as well as accumulation of CD43-positive inflammatory cells and activated HSCs in pericentral areas following infusion of Ang-Ⅱ, and concluded that liver injury is induced in this circumstance by oxidative stress, hepatic inflammation, and vascular damage[47]. It is considered[48] that Ang-Ⅱ acts by amplifying the general inflammatory response that follows the chronic liver injury, inducing reactive oxygen species (ROS) generation as well as inflammatory cytokines like interleukins (IL) -6 and -1, monocyte chemoattractant protein-1 (MCP-1), TGF-β1 and tumor necrosis factor-α (TNF-α). More complex connections and interferences are, however, occurring in real conditions, like the crosstalk between TNF-α and RAS in the TNF-induced plasminogen activator inhibitor-1 (PAI-1) production in human hepatocytes[49]. Accordingly, gene expression of RAS and that of PAI-1 are upregulated in the liver of patients with obesity and type 2 diabetes, and in non-malignant human hepatocyte cell lines, RAS-encoding genes are upregulated time-dependently by TNF-α while AT1-receptor blockade inhibits the TNF-induced PAI-1 production.

The mechanisms of inflammatory activation induced by Ang-Ⅱ are classic. AT1 receptor binding, with subsequent protein kinase C (PKC) activation followed by the intervention of intracellular signalling systems, like extracellular signal-regulated protein kinase (ERK) and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). c-Jun leads to the activation of nuclear factor-κB (NF-κB), preceded by the release of several of transcription factors, like activator protein-1 (AP-1) and signal transducer and activator of transcription (STAT), the final result being transcription and delivery of proinflammatory cytokines[50]. Additionally, other NF-κB-dependent inflammatory proteins such as cyclooxygenase-2 (COX2) and inducible nitric oxide synthase (iNOS) are upregulated by angiotensin. Jamaluddin *et al*[51] recently described in liver cells an alternate pathway for NF-κB activation similar to the signalling pathway that mediates antigen-induced lymphocyte proliferation by bridging T or B cell receptor. It can be, therefore, speculated that such a pathway can account for inflammatory changes that occur when "simple steatosis" turns to "steatohepatitis". Moreover, as activation of NF-κB is also followed by further stimulation of angiotensinogen transcription in hepatocytes by Ang-Ⅱ *via* the AT1 receptors[52], thus inducing expression of its own precursor and creating a biological "positive feedback loop", it is possible that this pathway represents one of the key factors that contributes to the vicious cycle of liver damage.

The key factors in promoting hepatic fibrosis are the HSCs, together with the portal myofibroblasts and cells of bone marrow origin which exhibit fibrogenic potential[53]. In liver fibrosis, the resident HSCs appears to be the primary source of myofibroblasts, although bone-marrow-derived cells can also contribute[54]. Chemokines attracting mononuclear phagocytes like MIP-1α (CCL3) and MCP-1 (CCL2) are considered as main profibrotic mediators, while TGF-β1 and Th2 cytokines (IL-4, IL-5, IL-13 and IL-21) have distinct roles in the regulation of tissue remodelling and fibrosis[55]. TGF-β1 is the best known pro-fibrotic cytokine, being stored in macrophages as an inactive homodimer that needs to be dissociated by several enzymes, like cathepsin, plasmin, integrins and MMPs, to bind to the specific receptors and to trigger intracellular intermediates (SMAD proteins) which induce procollagen Ⅰ and Ⅲ synthesis[56].

HSCs are the main source of extracellular matrix (ECM) in liver, residing in the space of Disse. When activated, HSCs express contractile, proinflammatory, and fibrogenic factors, migrate, secrete ECM, and regulate ECM degradation[57] by expressing MMPs. Activated HSCs are also a major source for additional proinflammatory mediators and cytokines and are able to *de novo* generate Ang-Ⅱ[58], being the key factor to maintain the vicious cycle which links inflammation to fibrosis. There is a consensus that Ang-Ⅱ and local RAS are major profibrotic agents in liver, inducing all the pro-fibrogenic properties of HSCs. Consequently, there are multiple points in which Ang-Ⅱ, acting on the AT1 receptors, increases the ability of HSCs to generate fibrosis, including stimulation of chemoatractant factors, activation of contractile and secretory properties of HSCs and imbalance of the production and removal of ECM.

Further, with the stimulatory effects of Ang-Ⅱ on MCP-1 and TGF-β1, with the implication of the AT1 receptor-mediated NF-κB-dependent pathway in this phenomenon, and its effects on TGF-β1 secretion and

activation, Ang-Ⅱ also enhances HSC's intracellular signalling by increasing SMAD levels and the nuclear translocation of phosphorylated SMAD with subsequent production of collagens, fibronectin and proteoglycans[59]. The contractile functions of activated HSCs, derived from intracellular smooth muscle actin expression, are also stimulated by Ang-Ⅱ which increases intracellular Ca^{2+}[47,60]; its proliferative capacity is also enhanced as showed recently by Liu *et al*[61] who observed that Ang-Ⅱ prompts HSC proliferation and DNA synthesis and also facilitates its contraction and collagen synthesis. These properties of HSCs are expressed through the mitogen-activated protein kinases (MAPK), a family of ubiquitous proline-directed, protein-serine/threonine kinases, which participate in signal transduction pathways that control intracellular events including apoptosis, cell growth, prostanoid formation, and other cellular dysfunctions when induced by oxidants or pro-inflammatory cytokines. These events are reversed by AT1 receptor blockade. By acting on the AT1 receptors in activated HSCs, Ang-Ⅱ also stimulates, *via* PKC intracellular signalling cascade, TIMP-1. This effect inhibits the activity of MMP which are responsible for collagen degradation and thus facilitates the progression of hepatic fibrosis[62].

Almost all the functions of HSCs, including the induction of proinflammatory cytokines, expression of NF-κB and production of ECM, are largely mediated by ROS generated by a nonphagocytic form of NADPH oxidase, which also plays a role in the inflammatory actions of Kupffer cells. NADPH oxidase is expressed at higher levels in response to cytokines and under inflammatory conditions, generating more free radicals[58], while Ang-Ⅱ also can induce supplementary production of ROS, providing a potentiating mechanism and creating an autocrine loop in which liver injury increases Ang-Ⅱ production that in turn perpetuates liver damage and fibrosis.

In opposition to the effects driven by the AT1 activation by Ang-Ⅱ, ACE2 and its product Ang-1-7, Mas receptors may counteract the adverse effects of Ang-Ⅱ in liver disease. Herath *et al*[63] examined the expression of these novel components of RAS and the production of Ang-1-7 in the bile duct ligated rats and observed that hepatic ACE2 gene and activity, plasma Ang-1-7 and Mas receptor expression increased after bile duct ligation. Moreover, perfusion experiments confirmed that bile-duct ligated livers produced increased Ang-1-7 from Ang-Ⅱ and this was augmented by ACE inhibition, leading to the conclusion that the RAS activation in chronic liver injury is associated with upregulation of ACE2, Mas and hepatic conversion of Ang-Ⅱ to Ang-1-7. These results support the theory that the presence of an ACE2-Ang-1-7-Mas axis in liver injury may moderate the effects of Ang-Ⅱ. Furthermore, Mas receptor antagonists have been tested in male Wistar rats subjected to sham-surgery or bile duct ligation[64]. Plasma renin activity and RAS components, as well as liver hydroxyproline and total TGF-β1 have been assessed, showing that renin activity, Ang-Ⅰ, Ang-Ⅱ and Ang-1-7 were progressively increased. Changes in RAS profile correlated with histological signs of fibrosis and deterioration in liver function while pharmacological blockade of the (Ang-1-7) receptor aggravated fibrosis with a significant elevation in hydroxyproline and total TGF-β1, suggesting that Ang-1-7 plays a protective role in hepatic fibrosis.

By observing in clinical conditions significant reduction of insulin resistance by both ARBs, as well as a moderate decrease of cytolysis in patients having NASH and mild-to-moderate hypertension, our study confirms, at least in part, these existing experimental data. There is, however, some unexplained issues, for example, why only telmisartan showed significant antifibrotic effects and why only this drug was able to improve the NAS score. Of course, a reasonable explanation could be the specific PPARγ modulatory activity of this ARB, but also other unique properties of this drug can contribute to this effect. As extensively discussed elsewhere[15], it seems that various ARBs have different "second-level" pharmacologic effects, unrelated to presence or absence of certain PPAR-modulating activity, as for example candesartan, which shows capacities to decrease liver fibrosis and diminish portal pressure in Child A cirrhotic patients[65], but do not have significant PPAR-modulating activity. It is subsequently possible that the better clinical results observed for telmisartan are driven through some undisclosed mechanism(s) that further studies will undoubtedly unveil.

Nevertheless, the limitations of our study are linked to the small number of patients, lack of a complementary analysis of plasma fibrosis markers and of serum leptin and adiponectin levels, and even a more complex evaluation of the lipid profile of the patients. Additionally, despite the fact that a rigorous analysis of antidislipidaemic effects of the two ARBs was out of our scope, we can, however, question as others[66] did, if the lipid-lowering effects observed for telmisartan, although statistically significant, have any clinical relevance and if the cytolysis improvement noticed for both ARBs has any impact for the clinical outcome of NASH. However, as the renin-angiotensin system plays a central role in IR and subsequently in NAFLD/NASH as the hepatic expression of MS, an attempt to block the deleterious effects of its overexpression seems correct and further studies are certainly needed to confirm weather an ARB can be a first-option drug for controlling IR, cytolysis and liver fibrosis in hypertension-associated NASH.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are well-recognized causes of progressive chronic liver disease leading to cirrhosis and hepatocellular carcinoma. These conditions, considered hepatic components of the metabolic syndrome (MS) are triggered by insulin resistance. To date, no therapy provided evidence of significant efficacy, and as a consequence, no approved therapeutic options are available worldwide.

Research frontiers

Although IR plays a pivotal role in NASH/NAFLD, potential therapies tested for these conditions treat only its consequences or try to eliminate excessive fat. As the renin-angiotensin system (RAS) plays a central role in IR and subsequently

in NAFLD/NASH, an attempt to block the deleterious effects of its overexpression seems an attractive breakthrough. By inhibiting RAS they can achieve an improvement of intracellular insulin signalling pathway, a better control of adipose tissue proliferation and adipokine production and a more balanced production for various cytokines. At the same time, by controlling the local RAS in the liver, they might be able to prevent at least fibrosis and to slow down the vicious cycle that links steatosis to necroinflammation. By targeting pancreatic effects of angiotensin they would be able to preserve an adequate insulin secretion and acquire a better metabolic balance.

Innovations and breakthroughs

This is the first human blinded trial evaluating the effects of telmisartan and valsartan in steatohepatitis that uses paired liver biopsies with NASH score (NAS) evaluation, simultaneously with cytolysis, IR and lipid profile assessment. Although serum aminotransferases did not normalized, telmisartan can reduce cytolysis by 30.28% and can improve IR by 42.63% consequently with a significant decrease of NAS and fibrosis scores and an amelioration of the lipid profile. Conversely, despite a significant reduction of cytolysis levels by 23.22% and of IR by 21.4%, valsartan did not improved liver histology (except steatosis) and had no effect on plasma lipids.

Terminology

ARBs are angiotensin receptor blockers, non-peptide compounds that have a binding affinity to the receptor AT1 of angiotensin thus inducing an irreversible or competitive blockade of the physiologic agonists.

Peer review

By observing in clinical conditions significant reduction of IR by both ARBs, as well as a moderate decrease of cytolysis, the study confirms that ARBs can act as an elegant tool for adequate correction of various imbalances that act consensually in steatohepatitis. ARBs not only can correct hypertension, but also can act on IR and the hepatic RAS, preventing and treating steatohepatitis as an end-organ effect of MS. On the other hand, ARBs can prevent collagen synthesis and further progression to cirrhosis. As equally cheap, effective and well-supported antifibrotic therapies are hard to be found we can predict that this property will put ARBs in the pole position for treating at least the liver fibrosis.

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BRIEF ARTICLES

Effect of B vitamin supplementation on plasma homocysteine levels in celiac disease

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Abstract

AIM: To investigate the effect of vitamin supplements on homocysteine levels in patients with celiac disease.

METHODS: Vitamin B6, folate, vitamin B12, and fasting plasma homocysteine levels were measured in 51 consecutive adults with celiac disease [median (range) age 56 (18-63) years; 40% men, 26 (51%) had villous atrophy, and 25 (49%) used B-vitamin supplements] and 50 healthy control individuals matched for age and sex. Finally, the C677T polymorphism of 5,10-methylene tetrahydrofolate reductase (MTHFR) was evaluated in 46 patients with celiac disease and all control individuals.

RESULTS: Patients with celiac disease and using vitamin supplements had higher serum vitamin B6 (P = 0.003), folate (P < 0.001), and vitamin B12 (P = 0.012) levels than patients who did not or healthy controls (P = 0.035, P < 0.001, P = 0.007, for vitamin B6, folate, and vitamin B12, respectively). Lower plasma homocysteine levels were found in patients using vitamin supplements than in patients who did not (P = 0.001) or healthy controls (P = 0.003). However, vitamin B6 and folate, not vitamin B12, were significantly and independently associated with homocysteine levels. Twenty-four (48%) of 50 controls and 23 (50%) of 46 patients with celiac disease carried the MTHFR thermolabile variant T-allele (P = 0.89).

CONCLUSION: Homocysteine levels are dependent on Marsh classification and the regular use of B-vitamin supplements is effective in reduction of homocysteine levels in patients with celiac disease and should be considered in disease management.

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Key words: Celiac disease; Homocysteine; Vitamin supplements

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INTRODUCTION

Numerous studies suggest that moderate hyperhomocysteinemia is an independent risk factor for atherothrombotic vascular disease[1,2] and recurrent venous thromboembolism[3]. Moderate elevations in total plasma homocysteine (tHcy) levels are commonly due to acquired deficiencies in B vitamin cofactors of homocysteine metabolism, including vitamin B6, folate, and vitamin B12[4]. Earlier trials have demonstrated that daily supplementation of folate or vitamin B12 could normalize the concentration of tHcy[5]. The American Heart Association has recommended screening for

hyperhomocysteinemia in patients with malnutrition and malabsorption syndromes[6].

Celiac disease is a typical example of a malabsorption syndrome conferring increased risk for various deficiency states, including folate and vitamin B12[7]. Even patients adhering to a strict gluten-free diet for years were prone to the development of various vitamin deficiency states, although they were in biopsy-proven remission[8]. Moreover, hyperhomocysteinemia is significantly more frequent in patients with newly diagnosed celiac disease than healthy controls and has been reported to improve after the institution of a gluten-free diet[9,10].

In view of these considerations, we investigated the effect of vitamin B6, folate, and vitamin B12 daily supplements on tHcy levels in patients with celiac disease.

MATERIALS AND METHODS

Between March, 2004 and November, 2005, 51 consecutive celiac disease patients attending a clinic for either initial or follow-up small intestinal biopsy (at least 12 mo after commencing a gluten-free diet) were included in the study. Patients were diagnosed with celiac disease based on the European Society of Pediatric Gastroenterology, Hepatology and Nutrition diagnostic criteria[11]. All patients carried either HLA-DQ2 (encoded by DQA1*0501 and DQB1*02 alleles) or HLA-DQ8 (encoded by DQA1*0301 and DQB1*0302 alleles) or both.

Patients were categorized into four groups: (1) newly diagnosed (untreated; all positive to IgA anti-transglutaminase and to IgA anti-endomysium antibodies and all had villous atrophy; n = 7); (2) persistent villous atrophy at follow-up due to dietary mistakes (non-compliant; all were positive to either IgA anti-transglutaminase or to IgA anti-endomysium antibodies; n = 7); (3) persistent villous atrophy at follow-up despite strict adherence to gluten free diet for at least 12 mo (refractory; all were negative to both serum antibody tests; n = 12); or (4) recovered villous architecture at follow-up biopsy (responsive to gluten free diet; six were positive to either IgA anti-transglutaminase or to IgA anti-endomysium antibodies; n = 25).

A dedicated dietician assessed the dietary behavior of all patients with celiac disease. Overall, twenty-five (49%) patients with celiac disease reported the regular daily use of vitamin supplements containing vitamin B6 (range 1-6 mg), folate (range 100-400 μg), and vitamin B12 (range 0.5-18 μg) for a median interval of 28 mo (range 18-84).

Blood samples were analyzed for serum vitamin B6, folate, vitamin B12, and fasting tHcy levels in all study patients within a median of 16 d (range 7-30 d) from small bowel biopsy.

In addition, serum vitamins and tHcy were measured in 50 healthy individuals, matched for age and sex, who served as controls. No individuals in the control group reported the use of vitamin supplementation. No patients with celiac disease or from the control group reported the use of drugs known to affect tHcy levels (e.g. diphantoine, methotrexate or theophylline). Finally, the C677T polymorphism of 5, 10-methylenetetrahydrofolate reductase (MTHFR) was evaluated in 46 patients with celiac disease and all controls. All controls and 90% of the patients with celiac disease were whites of European descent.

The Ethics Committee of the VU University Medical Center approved the study protocol, and all participants gave their written informed consent.

Laboratory analyses

Microparticle enzyme immunoassay method based on fluorescence polarization (IMX analyzer; Abbott, Chicago, IL, U.S.A.) was used to measure tHcy. Intra- and inter-assay CVs (coefficients of variation) were 2.1 and 5.1%, respectively[12]. Serum creatinine was measured by means of a modified Jaffé method and creatinine clearance was calculated according to the Cockroft-Gault method. Serum vitamin B6, folate, and vitamin B12 were measured with a competitive protein-binding assay (Dualcount Solid Phase Boil assay, DPC, Los Angeles, CA, USA; reference values 10-50 nmol/L, 6.8-39 nmol/L, and 150-700 pmol/L, respectively).

Genotyping of the MTHFR C677T SNP by polymerase chain reaction

A procedure using the commercial DNAzol reagent was applied to extract genomic DNA from peripheral blood. The region surrounding the MTHFR C677T SNP (dbSNP ID: rs1801133) was amplified using the polymerase chain reaction (with the primers 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' and 5'-AGGACGGTGCGGTGAGAGTG-3') as originally described[13]. PCR conditions were: 95℃ for 2 min, followed by: 35 cycles of 95℃ for 30 s, 55℃ for 30 s, 72℃ for 30 s, followed by a final extension of 5 min at 72℃. Since the MTHFR C677T SNP creates a restriction site for *Taq* I the 198 bp amplified fragment was digested by *Taq* I , followed by agarose gel electrophoresis and ethidium bromide staining, resulting in a 198 bp fragment when allele C is present and fragments of 171 bp and 27 bp when allele T is present.

Statistical analysis

Patients with celiac disease were once stratified into two subgroups with respect to the use of vitamin supplements [confirmed in 25 (49%) patients] and at another time into another two subgroups with respect to the presence of villous atrophy [documented in 26 (51%) patients]. The results were analyzed and compared between the subgroups and the controls. Continuous data having normal distribution are presented as mean (SD), and skewed data are presented as median (interquartile range). Categorical data are presented in frequencies and percentages. Comparison between groups was performed by means of the two-tailed t tests for data with normal distribution and the Mann-Whitney-U or Kruskal-Wallis tests when a non-parametric test was indicated. Pearson χ^2 or

Fisher's exact test when appropriate was used to compare categorical data. Independent associations between age, creatinine clearance, the carriage of MTHFR mutation, serum B6, folate, and B12 with tHcy were assessed by means of multiple linear regression analysis. For all statistical analyses, $P < 0.05$ was considered significant. All statistical tests were performed using the Statistical Software Package version 11.0 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Baseline clinical and biochemical characteristics of patients with celiac disease and controls are presented in Table 1. Patients with celiac disease had lower body mass index and creatinine clearance than healthy controls. When patients with celiac disease were stratified into subgroups according to their regular vitamin use, no differences appeared with regards to the body mass index or creatinine clearance (data not shown). The celiac-specific serological tests were also comparable between patients with celiac disease who did and who did not use vitamin supplements.

The median serum vitamin B6, folate, and vitamin B12 levels were significantly higher in patients with celiac disease on vitamin supplements compared to patients not using vitamin supplements and to healthy controls (Table 2). In accordance, patients with celiac disease taking vitamins had lower tHcy levels than patients who did not use vitamins and healthy controls ($P = 0.001$ and $P = 0.003$, respectively). Despite the absence of significant differences in B6, folate, and B12 status, tHcy was marginally, but significantly higher in celiac disease patients not using vitamins compared to controls ($P = 0.04$).

When patients with celiac disease were stratified into two groups according to small bowel histology, villous atrophy was found to be associated with higher tHcy levels ($P = 0.018$) while vitamin B6, folate, and vitamin B12 levels were comparable (Table 3). The finding was also accounted for by the celiac disease group not using vitamins ($P = 0.04$), but not for those using vitamins ($P = 0.2$).

Twenty-four (48%) of 50 controls and 23 (50%) of 46 patients with celiac disease carried the MTHFR thermolabile variant T-allele ($P = 0.89$). Genotype data were in Hardy-Weinberg equilibrium for the control subjects ($P > 0.05$). Individuals homozygous for the MTHFR C>T mutation was more frequently observed among controls than among patients with celiac disease (14% *vs* 7%; $P = 0.23$).

Multiple regression analyses showed that vitamin B6 and folate, but not vitamin B12, were significantly and independently associated with tHcy. Additional analyses revealed that the presence of celiac disease was associated with high tHcy levels independent of vitamin B6 or folate (Table 4). Additional adjustments for creatinine clearance, age, and MTHFR genotype did not affect this result (data not shown). As these results suggested that celiac disease affects tHcy independent of measured determinants of homocysteine, we analyzed the independent association with the severity of celiac disease, as assessed by the Marsh classification, and adjusted for vitamin B6 and folate. This analysis confirmed that the Marsh classification was an independent determinant of tHcy (Table 4). Again, additional adjustment for creatinine clearance, age, and MTHFR genotype did not affect the result.

Table 1 Baseline characteristics of patients with celiac disease and healthy controls

Characteristic	Healthy controls (n = 50)	Celiac disease (n =51)	P[1]
Median age (range), yr	48 (18-62)	56 (18-63)	0.09
Men, n (%)	22 (44%)	20 (39%)	0.7
Mean body mass index ± SD	25.0 ± 3.6	22.0 ± 2.7	< 0.001
Smoking, n (%)	4 (8.0%)	4 (7.8%)	1.0
Median creatinine clearance (range), mL/min	89.85 (54-124)	76.2 (40-156)	< 0.001

[1]Continuous data were compared by Student's t test (two-sided) and categorical data were compared by Fisher's exact test (two-sided).

DISCUSSION

This study demonstrated that patients with celiac disease using B vitamin supplements had lower tHcy levels than those who did not use B vitamin supplements. Second, even if villous atrophy persists, B-vitamin supplements can normalize B6, folate, B12 status, and tHcy levels. Finally, both the presence and the severity of celiac disease were determinants of tHcy levels, independent of measured B-vitamin status.

Serum B vitamins are essential cofactors in the metabolism of homocysteine, and vitamin B supplements can lower tHcy levels[14]. This finding is confirmed in our data from celiac disease patients. Patients with celiac disease not using vitamin supplements had higher tHcy levels than healthy controls despite comparable vitamin B6, folate, and B12 levels, and the presence of celiac disease as such was found to be a determinant of tHcy levels, independent of B6, folate, and B12 status. One explanation for this finding might be that tHcy is a more sensitive indicator of subtle B-vitamin deficiency compared to plasma B vitamin levels. Alternatively, unknown mechanisms related to celiac disease or variables not measured in this study, like riboflavin[15] or serine status[16], might play a role. A plausible explanation for the lower creatinine clearance in the celiac group might be the age factor, although this was not significantly different ($P = 0.09$). Creatinine clearance, however, did not appear to correlate with tHcy levels by further analysis.

Previous studies addressing the issue of B vitamin status and hyperhomocysteinemia in celiac disease have found similar results. In a study of 30 adult patients with celiac disease, tHcy levels were found raised in comparison with the general population[8]. In a prospective study, Saibeni *et al*[9] confirmed that hyperhomocysteinemia was significantly more frequent in patients newly diagnosed with celiac disease than

Table 2 Serum vitamin B6, folate, vitamin B12 and plasma homocysteine levels in patients with celiac disease (without and with regular vitamin supplements) and in healthy controls

Variable	Healthy controls n = 50	Patients with celiac disease not using vitamins n = 26	Patients with celiac disease using vitamins n = 25	P[1]
Median vitamin B6 (interquartile range), nmol/L	39.0 (30.2-54.2)	36.0 (21.0-77.2)	74.0 (28.0-183.5)	0.016
Median folate (interquartile range), nmol/L	9.7 (7.2-12.5)	12.1 (7.2-16.5)	29.9 (14.9-57.0)	< 0.001
Median vitamin B12 (interquartile range), pmol/L	234.5 (190.0-277.5)	230.5 (176.5-299.0)	342.0 (208.0-536.5)	0.017
Median homocysteine (interquartile range), μmol/L	9.7 (8.4-11.9)	11.0 (9.2-14.3)	7.1 (6.2-10.7)	0.001
Villous atrophy, n %		14 (54.0%)	12 (48.0%)	0.78

[1]Continuous data were compared by Kruskall-Wallis test and categorical data were compared by Fisher's exact test (two-sided).

Table 3 Serum vitamin B6, folate, vitamin B12 and plasma homocysteine levels in patients with celiac disease without (Marsh 0-Ⅱ) and with (Marsh Ⅲ) villous atrophy

Variable	All patients with celiac disease (n = 51)			Patients with celiac disease not using vitamins (n = 26)			Patients with celiac disease using vitamins (n = 25)		
	Marsh Ⅲ n = 26	Marsh 0-Ⅱ n = 25	P value[1]	Marsh Ⅲ n = 14	Marsh 0-Ⅱ n = 12	P value[1]	Marsh Ⅲ n = 12	Marsh 0-Ⅱ n = 13	P value[1]
Median vitamin B6 (interquartile range), nmol/L	54.5 (20.7-99.7)	60.0 (33.0-170.5)	0.26	34.0 (20.7-71.2)	61.5 (23.5-96.5)	0.59	72.0 (17.2-165.0)	91.0 (45.0-238.0)	0.47
Median folate (interquartile range), nmol/L	14.2 (7.2-33.9)	19.8 (11.5-28.9)	0.41	12.2 (6.2-16.1)	12.1 (7.7-19.1)	0.49	34.8 (11.7-75.4)	27.8 (18.6-36.1)	0.93
Median vitamin B12 (interquartile range), pmol/L	263.0 (195.2-433.0)	278.0 (177.5-353.5)	0.45	229.0 (173.7-295.5)	254.5 (171.7-303.0)	0.52	455.5 (256.7-916.2)	317.0 (180.0-380.0)	0.04
Median homocysteine (interquartile range), μmol/L	11.0 (8.1-16.1)	9.1 (6.7-10.7)	0.018	12.6 (10.5-17.4)	9.3 (9.1-13.4)	0.04	8.9 (6.1-13.2)	7.1 (6.1-8.8)	0.20
Use of vitamin supplements, n %	12 (46.1%)	13 (52.0%)	0.78[2]						

[1]Continuous data were compared by Kruskall-Wallis test; [2]Vitamin use versus no vitamin use frequency was compared by Fisher's exact test (two-sided).

in healthy controls[9]. Dickey *et al*[10] described the homocysteine and biomarker status of metabolically related B vitamins in patients with celiac disease (newly diagnosed = 35, non-responsive despite gluten free diet = 24, and recovered after gluten free diet = 41) and found that gluten exclusion in celiac disease improved folate status and normalized homocysteine levels. Our study demonstrates, in agreement with previous reports, that celiac disease is associated with elevated tHcy levels.

High tHcy concentrations in individuals with thermolabile MTHFR (T allele) have been reported among those patients with reduced plasma folate concentrations[17-19]. However, we found no significant differences when these two groups were compared (data not shown).

The consequences of higher tHcy levels in celiac disease may include an increased tendency to develop occlusive venous and arterial disease. Although this has been an understudied area, data are emerging that celiac disease confers an increased risk of vascular complications. The association of hepatic vein obstruction and celiac disease, for example, has been reported previously in five children[20] and in three adults[21]. Case reports have repeatedly described the concomitant presence of celiac disease, hyperhomocysteinemia, and thromboembolic events, like deep vein thrombosis[22], stroke[23], or pulmonary embolism[24]. The consequences of long-term (subtle) B vitamin deficiency may result in an increased propensity to develop malignancy[25], including lymphoma[26].

Table 4 The independent association of homocysteine with vitamin B6, folate, the presence of celiac disease, and villous atrophy, analyzed by multiple regression analysis

	Standardized coefficients beta	Significance
Presence of celiac disease		
Vitamin B6	-0.309	0.002
Folate	-0.366	0.000
Celiac disease	-0.194	0.053
Presence of villous atrophy		
Vitamin B6	-0.307	0.013
Folate	-0.363	0.004
Villous atrophy	0.294	0.018

Dependent variable: Homocysteine.

With respect to the effect of a gluten free diet, a previous study revealed the presence of elevated tHcy levels in patients with celiac disease on strict gluten free diet for ten years[8]. Others, however, have found that gluten free diet was able to normalize hyperhomocysteine levels[9,10].

Patients adhering to a strict gluten free diet are still prone to the development of various vitamin deficiency states[8]. Earlier, thiamin, riboflavin, and niacin contents of gluten free cereal products have been compared with their gluten containing counterparts[27]. Most gluten free

food has been found to provide lower amounts of at least one of these nutrients. In another report, 30 of 37 gluten free cereal products with available data on folate content contained lower amounts of folate compared with their gluten containing counterparts[28]. Of the 58 gluten free breads, pastas, and cold cereals, only 3 cold cereals were fortified with folic acid. None of the bread products or pastas were enriched with folic acid[28]. Consequently, the use of vitamin supplements next to gluten free diet in patients with long standing celiac disease has been advocated[8]. The favorable effect of vitamin supplements on B-vitamin and tHcy levels has been previously established and clearly appears to apply to patients with celiac disease[29,30].

Limitations of this study include the heterogeneity of study subjects that considerably affected the study design. Second, B-vitamin supplement treatment was not standardized. Third, the lack of follow-up data in our study did not allow us to reach conclusions regarding the effect of gluten free diet alone on tHcy levels. The recovery of villous atrophy following a gluten free diet has been earlier shown to normalize homocysteine levels[10]. However, treatment with a gluten-free diet and folic acid in case studies led to the variable improvement in homocysteine levels[31]. A useful study would be to randomize newly diagnosed patients with celiac disease to use B-vitamin supplements with a gluten free diet compared to a gluten free diet alone. Finally, it might be argued that current evidence suggests that there is no clinical benefit of the use of folic acid and vitamin B12 (with or without addition of vitamin B6) in patients with established vascular disease[32]. However, many other studies conducted over the past 25 years have provided ample support to the association of mild hyperhomocysteinemia with an elevated risk of atherosclerosis. In addition, several issues remain unresolved and require further investigation, including the proper dose of B-vitamin supplements, the proper duration of treatment, the timing of treatment (i.e. in primary or secondary prevention setting), and the implications of lower rates of stroke[33]. In any case, long-term B vitamin deficiency, which is clearly a risk associated with celiac disease, should be avoided and thus requires either monitoring of B vitamin levels and tHcy or standard treatment with moderate-dose B vitamin supplements.

In conclusion, celiac disease, the presence of villous atrophy, vitamin B6 and folate are independent determinants of tHcy levels. Use of B-vitamin supplements lowers tHcy levels, even if villous atrophy persists.

COMMENTS

Background

Celiac disease increases the risk for folate and vitamin B12 deficiency. Consequently, this can contribute to the development of hyperhomocysteinemia with its associated link to atherothrombotic vascular disease.

Innovations and breakthroughs

Although a gluten free diet can reverse deficiency states that can develop in patients with celiac disease, vitamin supplements have been shown to have an additional value in this context. In this study, the authors demonstrate that B-vitamin supplements to a gluten free diet has a protective role against the development of hyperhomocysteinemia, even when villous atrophy did not recover yet.

Applications

The regular use of B-vitamin supplements reduces the risk of hyperhomocysteinemia in patients with celiac disease and should be considered in daily clinical management.

Terminology

Hyperhomocysteinemia can develop secondary to B-vitamin deficiency states, which is likely to occur in patients with celiac disease. Not surprisingly, B-vitamin supplements contribute to the reversal of hyperhomocysteinemia in patients with celiac disease.

Peer review

This study contributes to what is already known about homocysteine status in celiac disease.

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Prevalence and risk factors of gastroesophageal reflux disease in Qashqai migrating nomads, southern Iran

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Abstract

AIM: To investigate the prevalence and risk factors of gastroesophageal reflux disease (GERD) symptoms in Qashqai migrating nomads with a different life style in Fars province, southern Iran.

METHODS: In summer 2006, 748 Qashqai migrating nomads aged 25 years or more were enrolled using a multiple-stage stratified cluster random sampling method. A questionnaire consisting of demographic characteristics, lifestyle and GERD symptoms (heartburn, regurgitation, chest pain, dysphagia, hoarseness and cough) as completed for each subject.

RESULTS: The questionnaire was completed in 717 subjects. The prevalence rate of GERD, defined as reflux occurring at least one time per week in the preceding year, was 33% (237 subjects). The prevalence was higher in older individuals (36.0% *vs* 28.9%, $P < 0.05$) and in those with other gastrointestinal complaints (51.0% *vs* 27.8%, $P < 0.001$), but not different in obese and non-obese subjects. It was also higher in those consuming fruits and vegetables more than once a week (36.2% *vs* 17.3%, $P < 0.001$). GERD had a positive correlation with smoking (42.1% *vs* 27.8%, $P < 0.001$), but a negative relation with non-alcoholic beverages. The association between GERD and non-steroidal anti-inflammatory drugs (NSAIDs) consumption was also significant (40.2% *vs* 25.4%, $P < 0.001$).

CONCLUSION: The prevalence of GERD (33%) is very high in Qashqai migrating nomads which may be due to a lower socioeconomic and educational level of these people and difference in the life style. Older age, frequent consumption of fruits and vegetables, smoking and NSAIDs are risk factors for GERD in this population.

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Key words: Gastroesophageal reflux disease; Prevalence; Risk factors; Nomads; 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]. It is a chronic disease that results from the abnormal exposure of esophageal mucosa to refluxed gastric contents[2]. Heartburn is the prominent symptom of GERD, impinging patients' quality of life[3]. Environmental factors are probably the main cause of GERD[4]. Various lifestyle factors are thought to be associated with gastroesophageal reflux symptoms (GERS), including body weight, nutrition, alcohol consumption, smoking and intake of non-steroidal anti-

inflammatory drugs and sleeping position. There are few population-based data on GERD in Asia[5-8]. Previously, we published on associated factors of GERD in Shiraz, southern Iran[9]. This cross-sectional study was carried out on Qashqai migrating nomads of Fars Province in southern Iran. They migrate between winter quarters near the Persian Gulf and summer quarters in the plateaus of Zagros mountains in the north of Fars Province. The life style of Qashqai migrating nomads differs greatly from urban lifestyle. They live with their animals, migrate more than 500 km in search of pasture for their domestic animals and do not bear the same stresses of urban population and do not consume the same food. They live in tents and are more active physically than urban people.

MATERIALS AND METHODS

Materials

This study was carried out on Qashqai migrating nomads from May to October, 2006. They lived in their summer quarters.

Methods

The multiple-stage stratified cluster random sampling method was used to select 748 subjects aged 25 years or more and of both genders. The project was approved by Ethics Committee of Shiraz University of Medical Sciences and a written consent was provided from each participant. A team of interviewers, who received training, completed the standardized questionnaire consisting of demographics, lifestyle and GERS for each subject. To determine the validity and reliability of the study, a pilot study was undertaken by invitation of 100 subjects to the local health centers, while the same interviewers and physicians completed the questionnaire again. The validity and reliability was determined as 70% and 82%.

Height and weight of each subject was also recorded. The following definitions were used to identify 2 principal GERD symptoms in the questionnaire including heartburn: a burning feeling in epigastrium rises through the chest in substernal area; regurgitation: liquid coming back into the mouth leaving a bitter or sour taste.

The frequency of reflux symptoms was defined. Subject was defined to suffer from GERD when she/he reported heart burn and/or acid regurgitation in the preceding year with a frequency of at least one time per week, irrespective of its severity or duration[10-12]. The study subjects were also asked to classify the intensity of their GERD as follows: mild, when easily tolerated; moderate: when the discomfort interfered with normal activities; and severe, when the subject was incapacitated and unable to perform normal activities. Sociodemographic variables included age, gender, marital status, biological characteristics, such as BMI [weight in kilogram in fasting state divided by square of height in meters, resulting in 3 categories of normal weight (< 24.9 kg/m^2), overweight (25-29.9 kg/m^2) and obese (> 30 kg/m^2)], dietary habits, smoking (cigarette or waterpipe), coffee and tea consumption and use of aspirin, non-steroidal anti-inflammatory drugs (NSAIDs) and acetaminophen.

Dyspepsia was defined as pain or discomfort and/or post-prandial fullness and/or early satiety in the past year.

Table 1 GERD in relation to demographic data and life style habits

	GERD *n* (%)			
	Present	Absent	*P*	OR
Sex: Male	93 (32.7)	191 (67.3)	0.887	0.98
Female	144 (33.3)	289 (66.7)		1.0
Age: ≥ 40	134 (36.0)	238 (64.0)	0.046	1.39
< 40	93 (28.9)	229 (71.1)		1.0
Marital status: Married	212 (34.2)	408 (65.8)	0.086	1.61
Single	22 (24.4)	68 (75.6)		1.0
BMI: Obese (> 30)	22 (38.6)	35 (61.4)	0.552	1.37
Over Wt.	55 (32.7)	113 (67.3)		1.06
Normal (< 25)	147 (31.5)	320 (68.5)		1.0
Hx[1] of headache: Yes	83 (49.1)	86 (50.9)	< 0.001	2.56
No	146 (27.4)	387 (72.6)		1.0
Hx of other GI[2] complaints: Yes	78 (51.0)	75 (49.0)	< 0.001	2.70
No	154 (27.8)	400 (72.2)		1.0

[1]Hx:Histroy; [2]GI: Gastrointestinal.

Statistical analysis

Information was put directly into a computer database under supervision of professional biostatistician. Statistical analysis was performed using the SPSS computer software package. $P < 0.05$ was considered to be statistically significant. All reported P values were two sided using χ^2 tests.

RESULTS

Seven hundred and forty eight Qashgai migrating nomads participated in the study and the interview questionnaire was completed in 717 subjects (response rate, 89%). Among the subjects, 284 (39.6%) were male and 433 (60.4%) were female. The mean age was 43.1± 14.2 years (max: 85, min: 18).

The prevalence rate of GERD, defined as reflux occurring at least one time per week in the preceding year, was 33% (237 subjects). Among them, 93 (39.2%) were male and 144 (60.8%) were female.

Table 1 shows the prevalence rate of GERD in relation to demographic data and life style habits. There was no difference between males and females regarding GERD, but GERD was more frequent in subjects older than 40 years ($P < 0.05$).

The prevalence of GERD was not related to marital status. In spite of a higher rate of GERD in obese subjects, the difference was not statistically significant ($P = 0.55$). We noticed that there was more history of headache in patients with GERD compared to those lacking suffering from such a present or past history of epigastric pain, distention, bloating and dyspepsia was also higher in subjects with GERD ($P < 0.001$).

Table 2 GERD in relation to dietary, smoking and drinking habits and medication

	GERD *n* (%)			
	Present	Absent	*P*	OR
Hx fried food: Yes	208 (31.8)	447 (68.2)	0.012	0.51
No	29 (47.5)	32 (52.5)		1.0
Veg/Fruit: ≥ 1 /wk	201 (36.2)	355 (63.8)	< 0.001	2.7
< 1/wk	23 (17.3)	110 (82.7)		1.0
Smoking: Yes	98 (42.1)	135 (57.9)	< 0.001	1.88
No	125 (27.8)	324 (72.2)		1.0
Tea: Yes	218 (32.5)	452 (67.5)	0.146	0.59
No	18 (45.0)	22 (55.0)		1.0
Water: Yes	221 (32.4)	460 (67.6)	0.133	0.54
No	15 (46.9)	17 (53.1)		1.0
Beverages: Yes	172 (30.7)	388 (69.3)	< 0.001	0.51
No	60 (46.5)	69 (53.5)		1.0
Yogurt drink: Yes	190 (31.4)	414 (68.6)	0.047	0.63
No	44 (41.9)	61 (58.1)		1.0
NSAIDs: Yes	144 (40.2)	214 (59.8)	< 0.001	1.97
No	91 (25.4)	267 (74.6)		1.0
Aspirin: Yes	5 (45.5)	6 (54.5)	0.380	1.70
No	233 (32.9)	475 (67.1)		1.0

Table 2 shows the prevalence of GERD in relation to dietary, smoking and drinking habits of the participants. The results indicated a lower prevalence of reflux in subjects having fried food ($P < 0.05$) and in those consuming fruits and vegetables less than once per week ($P < 0.001$).

There was an association between smoking habits and GERD; in those with history of cigarette and waterpipe smoking, GERD was more prevalent and in non-smokers, GERD was seen less often ($P < 0.001$).

The prevalence of GERD was not statistically in subjects with history of drinking tea ($P = 0.15$) or water ($P = 0.13$) with meals compared to those without such a history. On the other hand, subjects with history of drinking spirit dough (yogurt with water and mixed with salt) with meals had less reflux symptoms ($P < 0.001$, $P < 0.05$, respectively).

We noticed more symptoms in subjects taking NSAIDs and aspirin, but the difference was significant only for NSAID ($P < 0.001$).

DISCUSSION

In the present study, life style was associated with reflux symptoms in Qashqai migrating nomads in southern Iran. The average income and the level of education of nomads are lower than the national average. They consume more dairy products, like milk and yogurt, but rarely consume fast foods or readymade foods, because they live in tents together with their animals. They migrate more than 500 kms from summer to winter quarters and deprave many facilities of urban population.

In our study, the prevalence of GERD, as defined heartburn and/or acid regurgitation at least one time per week, was 33%, which is higher than most western societies in recent studies[13-15]. In a review of literature, Goh *et al*[16] found that the prevalence of heartburn in the West is 20%-40%, and 14% have weekly heartburn. This prevalence is higher than Asian population. The authors also found that the prevalence of GERD in Asia is either increasing or being recognized more frequently. In a study from urban and rural population of southern Iran, the prevalence of reflux symptoms occurring at least 3 times per week was 15.4%[9]. Hu *et al*[17] demonstrated that only 4.8% of Chinese population had GERD. On the other hand, Wong *et al*[18] in a study by telephone contact, reported a prevalence of 29.8%. Geographic differences in GERD prevalence are difficult to interpret, due to different case definitions and questionnaires used[19]. In our study, the higher prevalence of GERD may be related to lower socioeconomic status and/or lower education of subjects. Jansson *et al*[20], in a large population-based study, revealed in Norway, positive association between low socioeconomic status (based on occupation, education and maternal deprivation) and reflux symptoms. In a study in southern Iran, the prevalence of GERD was significantly higher in rural and illiterate subjects[9]. The relationship between lower educational level and the frequency of GERD which was discussed previously, probably reflects the action of certain unhealthy life style habits or less ability to modify such habits[14,21]. As shown in Table 1, the GERD prevalence was not different in males and females or different age groups. A population-based study in Olmsted county, Minnesota, did not demonstrate a relation between gender and GERD[13]. Some other studies demonstrated higher prevalence in females[7-9,14].

Table 1 also shows that body mass index and marital status were not associated to GERD. The association of BMI and GERD has remained inconsistent. Some studies showed no association between BMI and reflux symptoms[9,22], but some studies have shown higher prevalence of reflux symptoms in obese subjects[23].

Our study showed that history of headache was higher in subjects with GERD ($P < 0.001$). Subjects with GERD also had more other gastrointestinal symptoms like dyspepsia, abdominal pain and distention/bloating. The association between gastrointestinal symptoms and headache is frequently unrecognized. Meucci *et al*[24] found that patients with reflux-like and ulcer-like dyspepsia, the prevalence of migraine headache did not differ from that in control individuals, whereas a higher prevalence of migraine was noted in patients with dysmotility-like dyspepsia and in patients with nausea and vomiting alone. In the study by Aamodt *et al*[25] higher prevalence of headache was found in individuals with reflux, diarrhea, constipation and nausea. They suggested that headache sufferers generally are predisposed to gastrointestinal complaints.

Table 2 shows relationship between GERD symptoms and various food stuffs. Interestingly, we observed less reflux symptoms in subjects with history of higher consumption of fried food. In many studies, there is a direct correlation between dietary fat and GERD[23,26]. The inverse relationship with fried food should not be interpreted as a protective role for fat in diet. It is possible that nomads consume only small amounts of fried food with the traditional foods and this kind of food has a low volume anyway. However,

this question can not be answered within cross-sectional design of our study.

We also found higher prevalence of reflux symptoms in those who consumed fruits and vegetables more frequently (Table 2). Nocon *et al*[23] reported that consumption of fruits has a protective effect, while vegetable consumption had no significant association. Saberi-Firoozi *et al*[9] also reported protective effects of fruits and vegetables on reflux symptoms in Shiraz City, southern Iran.

In relation to lifestyle, smoking has often been cited as a risk factor for GERD. According to Nocon *et al*[23], smoking was a risk factor for GERD and was dose-dependent. Our results also showed more reflux symptoms in both cigarette and water pipe smokers. On the other hand, we found no association between reflux symptoms, tea and water drinking with meals or around meal time. Nomads do not consume coffee, but almost always drink tea. Drinks such as tea were reported to be linked to GERD, but this is controversial. Although tea has been shown to increase acid secretion but it does not contribute to GERD[27]. Chang *et al*[28] found no link between coffee or tea consumption and the incidence of GERD. There was also no affect of tea or coffee on GERD in Nocon *et al*'s study[23].

In relation to consumption of beverages, we found less frequent reflux symptoms in those who consumed beverage and churned sour milk (diluted yogurt with water and mixed with salt), which is a very popular non-alcoholic beverage in nomads. The cause is not clear, but it may be due to type and amount of soft drinks.

Some studies have observed an association between the use of aspirin or NSAIDs and presence of GERD[14,29], whereas others have not[13,30]. A higher consumption of NSAIDs and aspirin were visible in subjects with GERD, but our study was statistically significant for NSAIDs. One reason for this statistical significance for aspirin may be the small number of subjects who consumed aspirin (11 subjects consumed aspirin, 5 had GERD and 6 didn't).

In conclusion, the prevalence of GERD (33%) was very high in Qashqai migrating nomads which may be due to lower socioeconomic and educational level of these people and different lifestyle. The prevalence was higher in older individuals and in those with other gastrointestinal complaints, but not different in obese and non-obese subjects. It was lower in those consuming fruits and vegetables less than once per week. Smoking had positive correlation with GERD, but non-alcoholic beverage had an inverse correlation with GERD. The association between GERD and NSAIDs consumption was also significant. Future longitudinal studies and follow ups needed to clarify other possible risk factors and association with GERD.

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COMMENTS

Background

Symptoms of gastroesophageal reflux disease (GERD) represent one of the most frequent health problems in Western world. Various lifestyle factors are thought to be associated with GERD, including body weight, nutrition, alcohol consumption, smoking and use of non-steroidal anti-inflammatory drugs and sleeping position. There are few population-based data on GERD in Asia and none in nomadic populations.

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. Study participants were Qashqai nomads who migrate more than 500 kms from summer to winter quarters and deprave many facilities of urban population. Their average income and level of education are lower than the national average. They consume more dairy products, like milk and yogurt, but rarely consume fast foods or readymade foods, because they live in tents together with their animals. The prevalence of GERD was high (33%) in this population and older age, frequent consumption of fruits and vegetables, smoking and NSAIDs were suggested as the risk factors.

Applications

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

Terminology

Heartburn: a burning feeling in epigastrium that rises through the chest in substernal area. Regurgitation: liquid coming back into the mouth leaving a bitter or sour taste. GERD: occurrence of heart burn and/or acid regurgitation in the preceding year with a frequency of at least once a week, irrespective of its severity or duration.

Peer review

The study is well written and structured and the statistical management is good. Results are clearly exposed and discussion is complete. However, it may be interesting for developing countries where these kinds of populations still prevail.

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BRIEF ARTICLES

Point-of-care continuous ^{13}C-methacetin breath test improves decision making in acute liver disease: Results of a pilot clinical trial

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Abstract

AIM: To assess the role of the ^{13}C-methacetin breath test (MBT) in patients with acute liver disease.

METHODS: Fifteen patients with severe acute liver disease from diverse etiologies were followed-up with ^{13}C-MBT during the acute phase of their illnesses (range 3-116 d after treatment). Patients fasted for 8 h and ingested 75 mg of methacetin prior to the MBT. We compared results from standard clinical assessment, serum liver enzymes, synthetic function, and breath test scores.

RESULTS: Thirteen patients recovered and two patients died. In patients that recovered, MBT parameters improved in parallel with improvements in lab results. Evidence of consistent improvement began on day 3 for MBT parameters and between days 7 and 9 for blood tests. Later convergence to normality occurred at an average of 9 d for MBT parameters and from 13 to 28 d for blood tests. In both patients that died, MBT parameters remained low despite fluctuating laboratory values.

CONCLUSION: The ^{13}C-MBT provides a rapid, non-invasive assessment of liver function in acute severe liver disease of diverse etiologies. The results of this pilot clinical trial suggest that the MBT may offer greater sensitivity than standard clinical tests for managing patients with severe acute liver disease.

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Key words: Acute liver disease; Methacetin; Breath test; Fulminant hepatitis

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INTRODUCTION

Acute liver disorders, including severe acute liver disease, acute exacerbation of chronic liver disease, and fulminant hepatic failure comprise a major cause of morbidity and mortality from liver disease[1-3]. Decision-making in the treatment of patients with severe acute liver disease focuses on the early identification of patients that require liver transplantation. This identification is currently based on several clinical and laboratory parameters[4,5], including clinical assessments, serum liver enzymes, synthetic tests, and serum ammonia levels; however, these parameters lack accuracy in assessing liver function[4,6]. In addition, frequent assessment of liver function is important in the follow-up and management of patients with severe acute liver diseases[2,7-9].

Fulminant hepatic failure (FHF) is a medical emergency that affects approximately 2000 individuals each year in the United States, and is characterized by severe and sudden liver cell dysfunction resulting in coagulation disorders and hepatic encephalopathy in patients without previous liver disease[10,11]. FHF does not follow a homogenous course. The overall survival rates for patients with FHF are approximately 10%-30%[11]. Therefore, frequent assessment of liver function is critical. Given the significant morbidity and mortality associated with acute liver disease and FHF, there is considerable urgency in the early assessment

of the patients' clinical situation and disease severity. This early assessment affects patient placement (intensive care unit versus ward), initiation of supportive therapies, and listing for liver transplantation. Current predictors of survival in patients with FHF are far from optimal[12,13]. Thus, it is often problematic to make decisions concerning medical treatment and time of transplantation based on clinical and laboratory assessments[14-16]. A therapeutic dilemma arises from the need to provide expedient transplants to patients with a failing liver while avoiding unnecessary transplantations in patients that are likely to recover spontaneously[17-19].

Breath tests have been used for several decades in patients with acute and chronic liver disorders[20-22]. These tests are based on measuring exhaled metabolites of labeled substrates that have been ingested and metabolized by the liver. The amount and rate of appearance of the metabolite in the exhaled breath represent liver enzymatic activity, and a decline may serve as a measure of hepatic injury. The ideal substrate would be metabolized solely by the liver and, therefore, selectively reflect liver metabolic function. ^{13}C-phenylalanine (PheBT) and ^{13}C-galactose (GBT) breath tests provide liver-specific substrates that reflect the activities of two enzymes localized to the hepatocellular cytosol[23]. Both tests have been shown to accurately predict the severity of liver cirrhosis, and correlate well with the Child-Turcotte-Pugh score (CTP). The ^{13}C-caffeine breath test can detect chronic hepatitis B virus (HBV)-related fibrosis and has been used to monitor improvement in liver function in response to long-term lamivudine therapy[24]. Additional substrates include indocyanine green and aminopyrine; however, their use is limited due to a dependence on portal flow rate and low first-pass metabolism, respectively. Though each substrate measures only one metabolic pathway, studies have shown that each reflected overall hepatic metabolism[25-27].

Methacetin is rapidly metabolized by cytochrome P450 and lacks toxicity in small doses; thus, it is a good substrate for evaluating cytochrome P450 enzyme activity[28]. In healthy liver cells ^{13}C-methacetin is rapidly absorbed and, with a single O-dealkylation step, cytochrome P450 1A2 breaks it down into acetaminophen and $^{13}CO_2$. Like phenacetin, methacetin undergoes extensive first-pass clearance and any remaining labeled methacetin and metabolites are excreted in the urine[29]. In patients with histologically-proven chronic liver diseases, the rate of O-dealkylation of methacetin, assessed by the MBT, accurately reflects the degree of liver damage[30]. After oral administration of ^{13}C-methacetin, the recovery of $^{13}CO_2$ in the exhaled air over 30-min was significantly reduced in patients with chronic hepatitis or liver cirrhosis compared to controls. The non-invasive MBT reliably distinguished between early cirrhotic (Child A) and non-cirrhotic patients with 95.0% sensitivity and 96.7% specificity[31]. Although the MBT test is potentially useful, it has not been integrated into everyday clinical practice[32].

A major drawback in using traditional breath tests is the cumbersome method of isotopic ratio mass spectrometry. This method requires prolonged testing and analysis, imposes patient inconvenience, and delivers limited data points. In contrast, the BreathID® continuous online ^{13}C-methacetin breath test (MBT, Exalenz, Israel) is based on the measurement of $^{12}CO_2$ and $^{13}CO_2$ concentrations by molecular correlation spectroscopy (MCSTM) that can detect variations less than 1/1000 in the $^{13}CO_2/^{12}CO_2$ ratio[33,34]. A recent study showed its effectiveness in assessing liver function in patients with chronic liver disorders[35].

The aim of this study was to assess the role of the MBT for evaluation and follow-up in patients with severe acute liver disease. The data suggest that the MBT correlated with standard parameters and may serve as a more sensitive decision-making tool than conventional laboratory tests for follow-up of patients with severe acute liver disease.

MATERIALS AND METHODS

Patients

Between August, 2005, and September, 2007, all consenting adult patients with severe acute liver disease were recruited to the study group. Inclusion criteria consisted of age $\geqslant$ 18 years, and an acute elevation of transaminases $\geqslant$ 10 × ULN or elevation of bilirubin $\geqslant$ 10 × ULN with or without an elevated INR $\geqslant$ 1.5. All patients gave written informed consent to their participation in the study. Patients with significant encephalopathy that were unable to give informed consent were not recruited. The study was conducted in strict adherence to the principles of the Declaration of Helsinki, and was approved by the Institutional Review Board (IRB) committees and the Israel Ministry of Health Committee for Human Clinical Trials.

Biochemical analysis

All patients underwent a biochemical work-up which included a complete blood count and the activities of: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyltranspeptidase (GGTP), lactate dehydrogenase (LDH), albumin, total bilirubin, and prothrombin. Routine biochemical tests were performed with commercially available kits. Coagulation tests included INR and serum levels of coagulation factors 5 and 7. In cases where several blood tests were drawn in one day, statistical calculations were based either on the set of tests taken closest to the time of the breath test or the earliest and most complete set of tests for each date. Obstruction was ruled out in patients with elevated bilirubin.

Noninvasive evaluation of liver injury by breath test

Following an 8 h overnight fast, in the morning, patients were connected to the breath testing unit *via* a nasal cannula (IDcircuit^{TM}) and received 75 mg of N-(4-methoxy-^{13}C-phenyl) acetamide (Methacetin, Isotec,

USA) dissolved in 150 mL of water. As a solution, its absorption is not affected by gastric motility. Breath samples were automatically collected by the BreathID device (Exalenz, Israel) before and for 60 min after the labeled substrate was administered to the patient. The $^{13}CO_2/^{12}CO_2$ ratio in the breath samples was determined every 2-3 min. During the test period patients continued fasting and remained at rest to eliminate any variability in CO_2 production due to the ingestion of food or physical activity. Number of tests performed was not constant.

Analysis of breath test data

Results obtained from the device were expressed as percentage of administered dose of ^{13}C recovery (PDR) and the cumulative percentage of ^{13}C recovery over time (CPDR) at 20, 30 and 60 min after ingestion of methacetin, respectively, as well as the PDRpeak, and peak time. PDR refers to the rate at which the ^{13}C substrate is metabolized and exhaled expressed as percent per hour. PDR is based on the change in $^{13}C/^{12}C$ ratio for each patient, taking into account their specific parameters affecting overall CO_2, normalizing the results for any weight, height, dose and substrate type and purity[36,37]. CPDR is the numeric integral of PDR, and describes the total percent of substrate metabolized at any given accumulated time. Data are expressed in units of percent. The BreathID® device plots the PDR and CPDR in real time and the PDR peak value is then calculated.

Statistical analysis

The correlation of the Breath test parameters and blood test values was calculated using Pearson Correlation. A model of recovery based on exponential function [1-*const.**exp (*time_from_baseline*/*time_constant*)] for MBT returning to normal metabolic function and [1+*const.**exp (*time_from_baseline*/*time_constant*)] for blood test value decrease and return to normality was used to extract the time to recovery. Statistical analysis was performed only for patients who had at least 3 breath tests and 3 sets of blood tests over at least three different days. Generalized Reduced Gradient (GRG2) with nonlinear optimization was used to determine the functions constants.

Normal values

Normal values were determined using a group of 100 healthy volunteers (57 males and 43 females). These healthy controls were screened by medical history, physical examination, routine liver function tests, and abdominal ultrasound. All healthy volunteers had blood test results within normal limits. None had a history of active or previous liver disease, alcohol abuse, drug abuse, and none were taking medications. Based on the analysis of MBT results from 100 normal volunteers, a PDR Peak value of 30% h was considered to be normal. The average PDR Peak value was 35% ± 9 % h in healthy volunteers, with a minimum of 20% h and a maximum of 60% h. Because the blood test results were considered normal within a range of ± 30% of normal, we decided to set a similar range for normal PDR peak values. Furthermore, the normal ranges for blood tests typically take into account that a high value is critical, while the low value is less relevant. Therefore, the normal range is expressed as anything below the normal value + 30% (Table 1). Similarly, the PDR peak had critical significance at low values, but high values were less relevant. Therefore, we set the normal range as anything above 21% h (Table 1).

Table 1 Definition of normality for breath test parameters and blood tests

	Units	Normal values	± 30% of normal
Breath test	%/h	> 30	> 21
ALT	U/L	< 53	< 69
AST	U/L	< 60	< 78
INR	U/L	1.00	< 1.30
BIL	μmol/L	< 17	< 22

Table 2 Liver disease etiology and outcome

Etiology	Number of patients	Number recovered	Number deceased
Autoimmune hepatitis	5	4	1
Drug induced liver injury	3	3	0
Acute HBV	2	2	0
Acute HAV	2	2	0
Wilson's disease	1	1	0
Pregnancy associated	1	1	0
Space occupying lesion	1	0	1

RESULTS

Patient population

Fifteen adult patients had severe acute liver disease of diverse etiologies, including autoimmune hepatitis (n = 5), acute hepatitis A virus (HAV) (n = 2), acute HBV (n = 2), drug-induced liver injury (n = 3), neoplastic disease (n = 1), Wilson's disease (n = 1), and pregnancy associated liver disease (n = 1). The demographic and clinical characteristics are summarized in Tables 2 and 3. In two patients a grade 2 hepatic encephalopathy developed.

Patient outcomes

Thirteen patients ultimately recovered from their illness, based on both clinical and biochemical assessments (average recovery time 28 d, SD 22 d); two patients died (1 autoimmune hepatitis, 1 neoplastic disease); and none of the patients were transplanted. The first patient that showed an unfavorable course of illness died of sepsis before a transplant could be provided. The second patient died from metastatic cancer.

Correlation of MBT with clinical course

Figure 1 illustrates the method of comparing results from the breath test versus the blood tests along the clinical course of recovery. In all patients that eventually recovered, clinical improvement and normalization

Table 3 Liver tests parameters (mean ± SD)

Parameter	Female	Male	Overall
No. of patients	8	7	15
Weight (kg)	70.13 ± 20.03 (52-115)	82 ± 19.59 (55-115)	75.67 ± 20.07 (52-115)
Height (cm)	163.75 ± 6.69 (155-176)	175.57 ± 5.32 (167-184)	169.27 ± 8.47 (155-184)
BMI (kg/m^2)	25.91 ± 5.69 (19.95-37.13)	26.53 ± 5.86 (18.59-35.89)	26.20 ± 5.57 (18.59-37.13)
Age (yr)	33.50 ± 17.04 (18-61)	31.14 ± 14.02 (14-53)	32.40 ± 15.19 (14-61)
ALT (IU)	1162.38 ± 1926.69 (45-5795)	2807.86 ± 2468.82 (58-7535)	1930.27 ± 2278.22 (45-7535)
AST (IU)	996.88 ± 1306.56 (50-3928)	1475.71 ± 1104.09 (96-3294)	1220.33 ± 1198.8 (50-3928)
Bilirubin (μmol/L)	193.50 ± 207.39 (9-565)	232.86 ± 194.49 (41-599)	211.87 ± 195.27 (9-599)
INR (SI)	2.09 ± 0.90 (1.00-4.06)	1.80 ± 0.62 (1.26-3.02)	1.95 ± 0.77 (1.00-4.06)

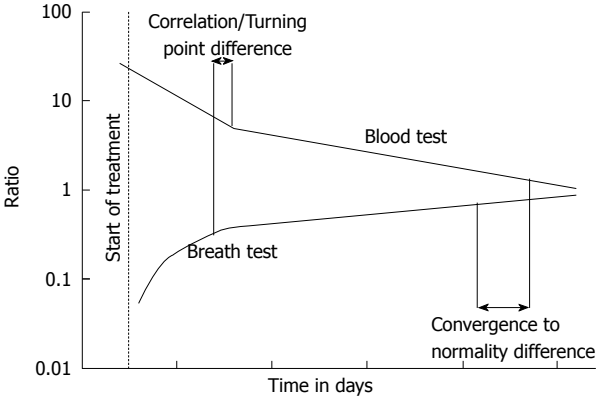

Figure 1 This is an illustration of typical breath test and blood test results plotted over the clinical time course. The wide green line indicates the range of normal values. As the patient recovered, test results converged toward normality.

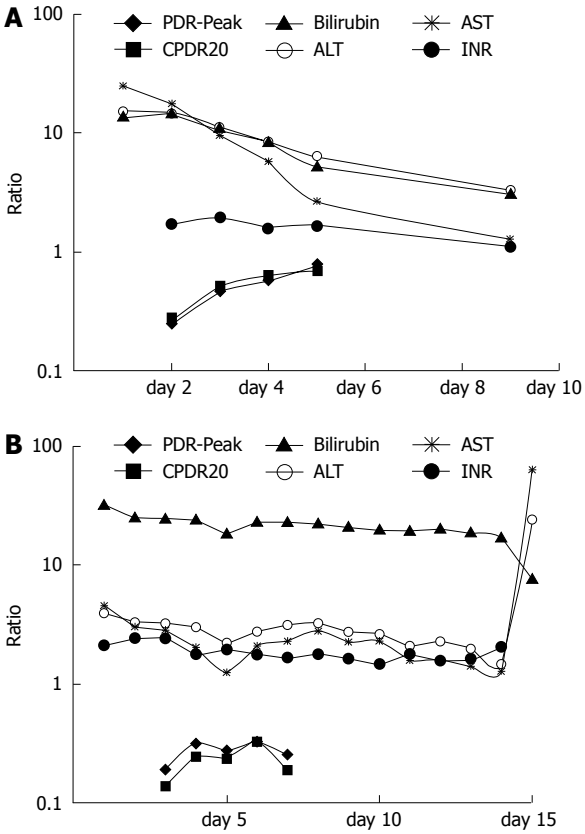

Figure 2 The clinical course of a patient with fulminant auto-immune hepatitis. A: Upon admission, aminotransferase levels were well above 10 × ULN, INR was increased, and breath test values were low. The patient received treatment with steroids and recovered rapidly. The breath test values normalized within 3 d, but blood tests began normalizing 2 d later; B: Despite therapy with steroids, this patient failed to improve, developed gram negative sepsis, and died. Note that despite stable blood tests, there was a lack of improvement in breath test values. The low breath test values may have been a negative prognostic factor in this patient and may have provided a rationale for early transplantation.

of biochemical parameters followed the progressive improvement of BreathID® ^{13}C-MBT scores (Figure 2A). In the two patients that died, breath test parameters deteriorated or failed to improve (Figure 2B). Overall, breath test parameters showed better correlation with clinical improvement than all other tests. Correlations between the breath test parameters and the blood test values varied between 0.2 and 0.7 (Table 4). The comparisons showed a trend for correlation; however, statistical significance was not reached due to the small number of patients and tests.

MBT results detect improvement in liver function prior to standard tests

The MBT indicated improvement in hepatic function prior to lab results. For this analysis, we defined 2 clinically relevant points. The first was the "point of stable improvement", which was defined as the point in time after which a continuous improvement was observed in patient MBT scores or blood tests results. The second was "convergence to normality," which was the point in time at which normal values were achieved (normal values are defined in the Methods). As shown in Table 5, the average point of stable improvement for MBT parameters occurred at 3 d, while for blood tests it occurred between 7 and 9 d after treatment. Later, the average convergence to normality for MBT parameters occurred at 9 d, while for blood tests it occurred within 13 to 28 d after treatment.

MBT parameters detect deterioration in liver function prior to other tests

In both patients that died, MBT parameters were extremely low at presentation and remained low throughout the course of the illnesses (despite medical therapy in the case of autoimmune hepatitis). In contrast, patient blood test parameters fluctuated during the course of the illnesses and finally deteriorated shortly before death (< 24 h).

DISCUSSION

The BreathID® ^{13}C-MBT is a rapid, non-invasive tool for the assessment of liver function in acute liver disease. This study showed that using the MBT to evaluate

Table 4 Correlation coefficients for blood test versus breath test parameters

	PDR peak	Peak time	PDR10	PDR15	PDR20	PDR30	PDR60	CPDR10	CPDR15	CPDR20	CPDR30	CPDR60
BIL	-0.556	0.394	-0.519	-0.544	-0.575	-0.588	-0.519	-0.456	-0.513	-0.540	-0.580	-0.595
ALT	-0.276	0.235	-0.258	-0.279	-0.293	-0.318	-0.403	-0.248	-0.266	-0.276	-0.306	-0.326
AST	-0.338	0.226	-0.306	-0.337	-0.358	-0.384	-0.451	-0.279	-0.311	-0.329	-0.362	-0.405
AP	-0.239	0.219	-0.308	-0.280	-0.268	-0.172	-0.110	-0.283	-0.301	-0.296	-0.282	-0.198
GGT	0.504	-0.344	0.510	0.538	0.507	0.418	0.203	0.340	0.449	0.483	0.497	0.438
LDH	-0.313	0.221	-0.313	-0.329	-0.329	-0.325	-0.314	-0.264	-0.302	-0.317	-0.333	-0.332
INR	-0.436	0.441	-0.412	-0.435	-0.452	-0.431	-0.386	-0.390	-0.420	-0.437	-0.458	-0.562
PTT	-0.565	0.239	-0.413	-0.509	-0.579	-0.632	-0.471	-0.360	-0.420	-0.471	-0.543	-0.598
PT%	0.628	-0.364	0.475	0.573	0.635	0.673	0.513	0.440	0.489	0.540	0.609	0.660
F-Ⅶ%	0.648	-0.828	0.720	0.737	0.723	0.613	0.581	0.661	0.694	0.715	0.725	0.688
F-Ⅴ%	0.576	-0.495	0.598	0.521	0.546	0.579	0.503	0.527	0.548	0.554	0.582	0.591

Results obtained from the device were expressed as percentage of administered dose of ^{13}C recovery (PDR) and the cumulative percentage of ^{13}C recovery over time (CPDR) at 10, 15, 20, 30 and 60 min after ingestion of methacetin, as well as the PDR peak, and peak time. PDR refers to the rate at which the ^{13}C substrate is metabolized and exhaled expressed as percent per hour. PDR is based on the change in $^{13}C/^{12}C$ ratio for each patient. CPDR is the numeric integral of PDR, and describes the total percent of substrate metabolized at any given accumulated time. Data are expressed in units of percent. ALT: Alanine aminotransferase; AST: Aspartate amino transferase; GGT: Gamma glutamyl transpepetidase; AP: Alkaline phosphatase; F-Ⅴ, F-Ⅶ: Factor 5 and 7; PDR: Percent dose recovery at different time points in minutes; CPDR: Cumulative percent dose recovery at different time points.

Table 5 Blood tests versus breath test parameters (mean, SD)

	Point of stable improvement (days after treatment)	Convergence to normality (days after treatment)
Breath test	2.85, 2.23	8.89, 11.5
ALT	7.33, 11.06	28.24, 21.69
AST	7.62, 11.44	12.96, 6.96
INR	8.62, 12.1	16.02, 24.71
BIL	7, 10.61	23.63, 22.61

patients with acute severe liver disease enabled detection of improvement 4-6 d earlier than the currently used laboratory tests, irrespective of disease etiology. Furthermore, breath test results indicated normalization of liver function 4-19 d earlier than blood test values in patients that recovered from their illnesses. The data of the present study suggests that the MBT may serve as a more sensitive decision-making tool than standard test parameters in the setting of severe acute liver disease.

In fulminant hepatic failure (FHF) an early prognosis is essential in determining the need and appropriate timing of orthotopic liver transplantation (OLT). Several reports have described laboratory parameters that served as predictive criteria. Since its publication by O'Grady and colleagues in 1989[38], the King's College Criteria have been widely used to define patients with poor prognoses using pH, thrombin time, serum creatinine, and bilirubin levels. Recently, the Acute Physiology and Chronic Health Evaluation (APACHE Ⅱ) score was described for determining prognosis in the setting of FHF. The APACHE Ⅱ used a combination of both clinical and laboratory parameters and its sensitivity was comparable to that of the King's College Criteria[39]. A recent study in 120 consecutive patients with FHF investigated the prognostic efficacies of King's College criteria, Clichy's criteria, the Model for End-Stage Liver Disease (MELD), and the Pediatric End-Stage Liver Disease (PELD). MELD scores were significantly higher in patients that died compared to those that survived without OLT. Logistic regression analysis yielded concordance statistics that were significantly higher for MELD (0.95) and PELD (0.99) compared to King's College (0.74) and Clichy's criteria (0.68). In a Cox model analysis, the data included patients that received transplants and censored the time from admission; this analysis showed the concordance statistics for MELD (0.77) and PELD (0.79) remained significantly higher than that of King's College criteria, but not higher than that of Clichy's criteria[40]. However, the MELD and PELD were not effective for follow-up on a daily basis, thus they were not useful for decision-making in the acute setting. The lack of an effective measure of liver function in the acute setting makes it difficult to reach an appropriate clinical decision in hepatology[41,42].

Noninvasive tests were used previously to assess liver function in acute liver disease. In 1993, Luketic *et al*[43] demonstrated that hepatic lidocaine metabolism was useful in the selection of patients for liver transplantation[44]. The study evaluated hepatic conversion of lidocaine to its primary metabolite, monoethylglycin exylodide, and compared the results to liver histological findings in 225 patients with chronic hepatitis. A decline in monoethylglycinexylodide production was correlated with worsening liver histological findings. A further stepwise decline in monoethylglycinexylodide production was correlated with a worsening Child class score. In contrast, no relationship was observed between liver histological status and serum transaminases (AST or ALT), bilirubin, albumin, or prothrombin time. Thirty-five patients underwent a follow-up histological evaluation and monoethylglycinexylodide testing after receiving at least 6 mo treatment for chronic hepatitis (interferon for hepatitis B and C and corticosteroids for autoimmune hepatitis). The change in monoethylglycinexylodide production was linearly related to the change in the Knodell histological index.

MBT has several advantages compared with the

above tests. It is a noninvasive, easily preformed, bedside test and is not associated with patient discomfort. It provides real time results and does not require any expertise for data analysis. It has no known side effects and is not limited by patient-specific parameters. Finally, the test is sensitive to minor changes in liver metabolism, thus enabling daily follow-ups in the acute setting.

The cause of acute liver failure (ALF)[6] is a major determinant of its outcome. Thus, the prognosis for ALF due to acetaminophen (paracetamol) overdose is relatively good, but the mortality rate for ALF from other causes may be significantly higher. In the current study, only 2 patients (13%) had acute liver damage due to paracetamol, but a far greater percent presented with acute autoimmune hepatitis. This may be explained by the observation that paracetamol is the etiological agent for a large percentage of ALF cases in England and the US, but it accounts for far fewer incidents in Europe and Israel (unpublished results). In a study performed in Spain, 267 cases of ALF were analyzed retrospectively. Acetaminophen overdose accounted for only 2.2% of the patients and overall survival was 58%. Liver transplants were performed in 150 patients, with a survival of 69%. Patients that fulfilled the criteria, but were not provided transplants due to contraindications, had a survival rate of only 7.8%. Those that did not fulfill the transplant criteria had a 85.5% survival rate[45]. The high survival rate in our group of patients might be explained by the fact that only 2 patients (13%) fulfilled the transplant criteria. One (autoimmune hepatitis) patient died from sepsis before transplantation was possible, and the other patient began to recover spontaneously a few hours before the scheduled transplantation. In this patient, the breath test made it possible to follow liver function even after correction of coagulopathy with fresh frozen plasma in preparation for liver transplantation.

Orthotopic liver transplantation is the most definitive solution to the problem of sudden hepatocyte loss. However, the selection of a lifetime surgical solution for an acute, potentially self-limiting problem is a stressful decision for physicians. Several obstacles to successful transplantation may arise, including transporting a patient with cerebral edema safely to the transplantation center, securing funding in a timely fashion, and obtaining a suitable organ rapidly. The overall one year survival rate of patients with acute liver failure that undergo liver transplantation exceeds 60 percent. However, due to the logistic hurdles that must be overcome, it is estimated that currently only 10 percent of patients with acute liver failure receive a liver graft. Subsequently, any test that may help in determining recovery at an earlier stage, thus deferring the need for transplantation, is of great value.

The current study had several limitations. The cohort only included 15 patients. The requirement for obtaining informed consent excluded patients with encephalopathy; thus, patients on that end of the spectrum were not assessed. Indeed, only two patients in this cohort displayed an indication for liver transplantation. In order to demonstrate an effect of MBT on decisions regarding liver transplantation, a larger group of patients with fulminant liver failure must be studied. However, even in the limited number of cases studied here, we found the MBT test offered an advantage as a decision-making tool because improvement in MBT parameters provided a rationale for avoiding unnecessary liver transplantation in one of these patients.

In summary, the results of the present preliminary study suggest that a point of care, on-line breath test system may provide an important tool for decision-making in clinical hepatology in the setting of acute severe liver disease. Further large scale studies are required for assessment of the general utility of this test.

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BRIEF ARTICLES

Protective effects of electroacupuncture on acetylsalicylic acid-induced acute gastritis in rats

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Abstract

AIM: To investigate the protective effects of electroacupuncture (EA) pretreatment on acetylsalicylic acid (ASA)-induced ulceration in rats.

METHODS: We randomly divided 72 rats into three groups including control (administered with distilled water), ASA group (administered 100 mg/kg ASA) and EA group (administered EA + 100 mg/kg ASA). Each rat was fasted for 18 to 24 h before experimentation, and lesion scores, gastric acidity, cyclooxygenase (COX)-1 and -2 mRNA levels, and total nitric oxide (NO) concentration were measured.

RESULTS: The lesion scores of the EA group were significantly lower than those of the ASA group. Gastric acidity of the ASA and EA groups was reduced compared to the control group. COX-1 and -2 mRNA levels were significantly increased in the EA group as compared to the control and ASA groups, and NO levels were also significantly increased in the EA group as compared to the ASA group.

CONCLUSION: These results suggest that EA-mediated protection against ASA-induced ulceration in rats may occur *via* gastric defense components.

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Key words: Electroacupuncture; Acute gastritis; Cyclooxygenase; Nitric Oxide

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used as anti-inflammatory and analgesic agents[1,2]. Unfortunately, they often cause gastrointestinal injury in gastric lesions by inhibiting COX; however, the detailed mechanism remains unclear[3]. Thus, effective strategies are required to protect the gastrointestinal mucosa. Acupuncture has been used to treat a wide variety of conditions, including fever, inflammation, infection and, especially, stomach diseases[4,5]. Therefore, we have investigated the protective effects of acupuncture against NSAID-induced ulceration in a rat model. Oxidization of arginine by nitric oxide synthase (NOS) creates the volatile gas NO, which has numerous physiologic properties, including regulation of inflammation and a role in gastric mucosal defense[6,7].

Acupuncture has been used to treat different gastrointestinal disorders in East Asian countries. ST-36 (stomach-36) is the common point for the treatment of gastric symptoms, such as nausea and vomiting, suggesting that acupuncture at ST-36 may stimulate gastrointestinal motility in humans[8]. A previous study has demonstrated that electroacupuncture (EA) at ST-36 inhibits acid secretion in conscious dogs and that naloxone reversed the EA-induced inhibition of acid secretion[9]. Other studies reported that EA stimulation at ST-36 increases

gastric acid secretion in rats, and sectioning of the sciatic nerve blocked the acupuncture induced acid secretion[10]. Many studies reported improvement of gastrointestinal symptoms, but the mechanism of the beneficial effects of acupuncture remains to be investigated.

In this study, we investigated whether pretreatment with EA could protect against 100 mg/kg ASA-induced ulceration in rats by regulating acidity, COX mRNA and NO levels of gastric defensive components.

MATERIALS AND METHODS

Animals

Seventy-two adult male Sprague-Dawley rats (Orient Bio Inc., Korea) weighing 170-210 g were included in this study. The rats were randomly divided into three groups: control (fasted 18-24 h and orally administered 1 mL DW), ASA (fasted 18-24 h and orally administered 100 mg/kg ASA) and EA (fasted 18-24 h, treated with EA for 30 min, and then immediately orally administered 100 mg/kg ASA). Rats were housed at 22 ± 1℃ and 55% ± 10% humidity on a 12 h light/12 h dark cycle with free access to food and water. All experimental procedures were approved by the Laboratory Animal Center at the Korea Institute of Oriental Medicine. After the rats were allowed to adjust to their new environment for three to four days, they were kept in cages with raised mesh bottoms and deprived of food, but allowed free access to tap water for 18 to 24 h.

Electroacupuncture stimulation

EA was applied *via* bilateral electrical stimulation at the ST36 acupuncture point using a pair of bipolar stimulation electrodes in rats under 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane (isoflurane) anesthesia. The electrical stimulation condition was biphasic pulses with 10 Hz frequency, 1 mA current and a duration of 0.1 ms.

Lesion score measurement

Like the EA group, the ASA group was anesthetized for 30 min; during this 30-min period, the EA group also received EA at ST-36 for 30 min before receiving 1 mL ASA (100 mg/kg) under isoflorane anesthesia. The control group was anesthetized for 30 min and administered 1 mL DW. Four hours later, all rats were sacrificed under i.p. pentobarbital (50 mg/kg, HANLIM PHARM. CO., Ltd, Korea) anesthesia to score the lesions of each stomach after inflation with 5 mL phosphate-buffered saline (PBS, pH 7.4). We utilized direct scanning of stomach samples to measure the gastric lesions with public domain image processing and analysis software developed at the National Institutes of Health, USA. The PC version of this program (2.15 MB), known as Scion Image, is freely available on the internet from Scion Corporation (http://www.scioncorp.com, Scion Image for Windows, Release Alpha 4.0.3.2)[11].

Determination of gastric acidity

Gastric acidity was measured using the pylorus ligation technique. Briefly, the abdomen was incised, and the pylorus was ligated under isoflurane anesthesia. Rats were continuously anesthetized for 30 min. The EA group received EA at ST36 for 30 min under anesthesia. Afterwards, the control group received distilled water, and the ASA and EA groups received ASA (100 mg/kg). Four hours after completion of all procedures, each group of rats was sacrificed under i.p. pentobarbital (50 mg/kg, HANLIM PHARM. CO., Ltd, Korea) anesthesia, and the gastric juices were collected. Gastric juices were centrifuged at 5500 r/min for 10 min and used for the following measurement. Titratable acidity was determined in a 200 μL aliquot of the gastric juice supernatant with 0.1 mol/L NaOH using phenol red as an indicator. Results were expressed as μEq/200 μL.

Analysis of COX-1 and COX-2 mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

Stomach samples for RT-PCR were immediately frozen in liquid nitrogen and stored at -70℃ until use. Total RNA was extracted from the stomach samples that were pooled from three rats using RNA Bee (Tel-Test Inc., TX, USA) and isopropanol-chloroform extraction. After isolating the RNA, we performed RNA quantitation using a spectrophotometer (NanoDrop ND-1000, NanoDrop, Texas, USA). Total RNA was reverse transcribed using a RevertAid™ First Strand cDNA Synthesis Kit (Asuragen Inc., Texas, USA) according to the manufacturers' instructions. β-actin levels were constant in all groups. The resulting cDNAs were amplified by PCR using β-actin, COX-1 or COX-2 specific primers, 10 mol/L dNTP, 10X Taq buffer and Taq. PCR cycling conditions consisted of 45 s at 94℃, 45 s at 59.3℃ and 1 min for 72℃ for β-actin and for 30 s at 94℃, 30 s at 53.6℃ and 45 s at 72℃ for COX-1 and -2. The sense and antisense primers were 5'-TGT GATGGTGGGAATGGGTCAG-3' and 5'-TTTGAT GTCACGCACGATTTCC-3' for β-actin (514 bp); 5'-GC CTCGACCACTACCAATGT-3' and 5'-AGG TGGCAT TCACAAACTCC-3' for COX-1 (167 bp); and 5'-TAC CCGGACTGGATTCTACG-3' and 5'-AAGTTGGTG GGCTGTCAATC-3' for COX-2 (214 bp). PCR was conducted for 28 cycles for COX-1 and -2, and 30 cycles for β-actin. Amplified DNA was electrophoresed in a 1.5% agarose gel. Fragment size was assessed by comparison to a 100 bp DNA marker (Solgent, Korea).

Determination of NO concentration

The gastric mucosa of each rat was removed by scrubbing and then weighed. NO content was determined using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Co, MI, USA). Briefly, samples were homogenized in PBS (pH 7.4), centrifuged at 10000 r/min for 20 min, and then ultra-filtered using a 30 kDa molecular weight cut-off filter (Millipore Corporation, MA, USA). Nitrate in the prepared samples was converted to nitrite using nitrate reductase and subsequently coupled with N-1-naphthyl-ethylene diamine to give a colored product whose concentration was measured colorimetrically at a wavelength of 540 nm.

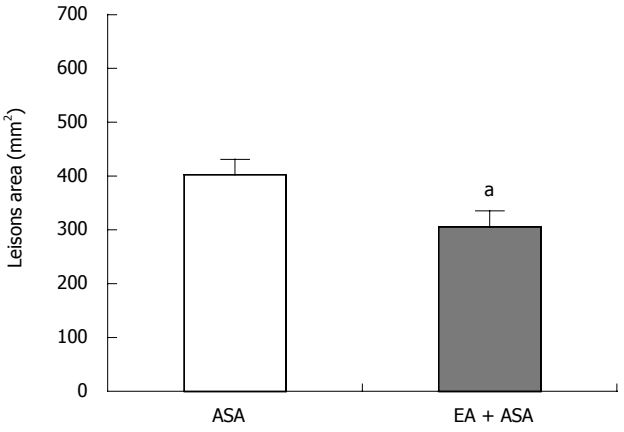

Figure 1 Protective effect of pretreatment of EA on ASA-induced gastric lesions in rats. Data are presented as the mean ± SE (n = 8 each group). [a]P < 0.05 compared with ASA group. ASA: Acetylsalicylic acid (100 mg/kg); EA: electro-acupuncture.

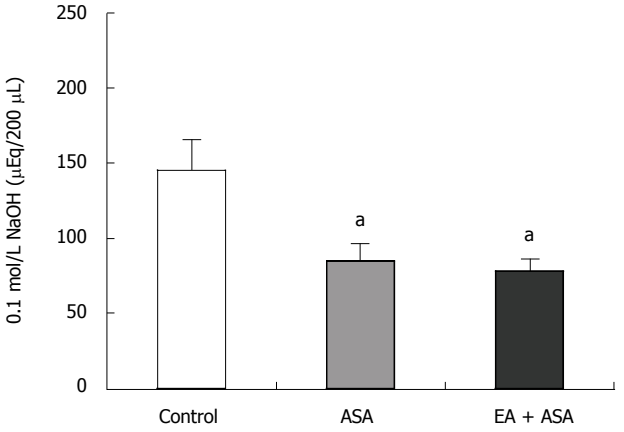

Figure 2 Determination of gastric acidity was measured in terms of each group. Rats were treated with or without 100 mg/kg ASA after anesthesia and pretreatment of EA on ST-36 for 30 min. Data are presented as the mean ± SE. [a]P < 0.05 compared with Control group. Control: Distilled water (n = 5); ASA: Acetylsalicylic acid (100 mg/kg, n = 7); and EA+ASA: Electro-acupuncture on ST-36 + ASA (n = 8).

Statistical analysis

All data were expressed as mean ± SE using SPSS for windows (version 11, SPSS, Inc., Chicago, USA). Differences between means were analyzed using one-way analysis of variance (ANOVA) with the Tukey post-hoc test for multiple comparisons as well as Student's t-test, $P < 0.05$ was considered significant.

RESULTS

EA decreased lesion severity

The lesion scores of the EA group (305.55 ± 29.67 mm^2, n = 8) were significantly lower ($P < 0.05$) than those of the ASA group (402.64 ± 28.25 mm^2, n = 8), as shown in Figure 1. Thus, EA pretreatment significantly protected against gastric lesions induced by 100 mg/kg ASA.

Effects of EA on acid secretion

Figure 2 shows the gastric acidity for each group: 145.2 ± 19.9 μEq/200 μL for the control group (n = 5), 84.86 ± 11.03 μEq/200 μL for the ASA group (n = 7), and 78.13 ± 8.01 μEq/200 μL for the EA group (n = 8). The gastric acidity of both the ASA and EA groups was significantly lower than that of the control group ($P <$ 0.05 in both groups).

COX-1 and COX-2 levels

The level of COX-1 mRNA in the ASA group was significantly decreased at one and two hours after administration of 100 mg/kg ASA (Figure 3 columns B and C) and then increased at four hours after administration (Figure 3 column D) compared to the control group (column A). Expression of COX-1 mRNA in the EA group was significantly increased at one, two and four hours after administration of 100 mg/kg ASA (Figure 3, columns E, F, G) compared to the control group. COX-2 mRNA levels were slightly up-regulated in all tested conditions and time points, except for the strong up-regulation observed in the EA group at four hours after administration of 100 mg/kg ASA (Figure 3).

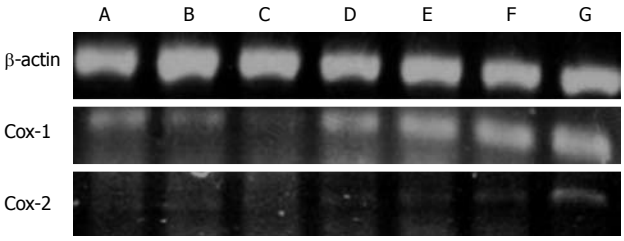

Figure 3 Expression of COX-1, 2 mRNAs in the rat stomach after oral administration of DW, ASA (100 mg/kg) or ASA (10 mg/kg) with EA pretreatment (n = 3 each group). A: Control; B: one hour after administration of ASA (100 mg/kg); C: Two hours after administration of ASA (100 mg/kg); D: Four hours after administration of ASA (100 mg/kg); E: One hour after administration of ASA (100 mg/kg) with EA pretreatment; F: Two hours after administration of ASA (100 mg/kg) with EA pretreatment; G: Four hours after administration of ASA (100 mg/kg) with EA pretreatment. ASA: Acetylsalicylic acid; EA: Electroacupuncture.

Determination of NO concentration

The total nitrate (NO_3^-) and nitrite (NO_2^-) concentrations of control, ASA and EA groups were 44.48 ± 2.06 μmol/L, 19.34 ± 1.05 μmol/L and 32.96 ± 1.68 μmol/L, respectively (Figure 4). These results demonstrate that the total NO in the EA group was significantly higher than that of the ASA group, but still significantly lower than that of the control group.

DISCUSSION

Acupuncture has been used to treat stomach diseases with few adverse effects. In this study, we found that the lesions scores of the EA group were significantly lower than in the ASA group. Although hypotheses abound regarding NSAID-induced gastric damage, the exact mechanism remains unclear.

The acidity of gastric secretions is important for modulating gastric defenses because acid can interfere with restitution, resulting in the conversion of superficial injury to deeper mucosal necrosis[12]. Additionally,

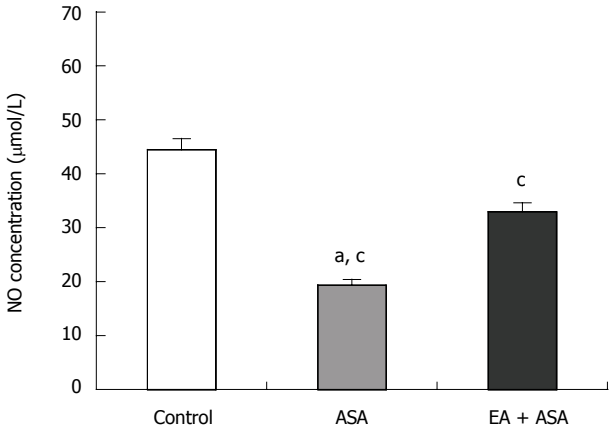

Figure 4 Total nitric oxide (NO) concentration of each group. Values are each a mean ± SE (n = 5 each group). $^{a}P < 0.05$ compared with Control; $^{c}P < 0.05$ compared with ASA group. Control: Distilled water, ASA: Acetylsalicylic acid (100 mg/kg); EA: Electro-acupuncture.

because several growth factors (e.g. fibroblast growth factor) that are important for maintenance of mucosal integrity and repair of superficial injury are acid-labile, the presence of acid in the lumen can limit the contributions of these factors to mucosal defense and repair[13]. Decreased acidity in the ASA and EA groups in the present study might have resulted from compensation for the destruction of gastric defense mechanisms by exposure to 100 mg/kg ASA.

The expression of COX-1 mRNA was increased in the EA group, but decreased in the ASA group. The expression of COX-2 mRNA in the EA groups gradually increased over time. This study thus showed that both COX-1 and COX-2 are inducible, which contradicts previous studies[14]. The reduction of gastric blood flow following NSAID administration appears to primarily arise from COX-1 inhibition, but neutrophil adherence following NSAID administration appears to be due to COX-2 suppression[12]. Administration of a selective COX-2 inhibitor, but not a COX-1 inhibitor, has been shown to induce neutrophil adherence[15]. A previous study reported that treatment with a selective COX-2 inhibitor aggravated dextran sulfate sodium-induced inflammation in the colon and that endogenous prostaglandins (PGs) were involved in the mucosal defense against chemo-induced ulceration; this response was produced by COX-1 in the early phase and COX-2 in the late phase[16]. Thus, the presently observed inducible expression of COX-1 in the early phase and COX-2 in the late phase suggests that EA pretreatment might up-regulate the mucosal defense mechanism.

NO in the vascular endothelium mediates various biological actions under physiological conditions[17] and also plays an important role in the modulation of gastric mucosal integrity by interacting with other protective mediators[18,19]. Several studies have reported that NO, or NO donors, stimulate PG production in various organs and cells[20-22] and up-regulate acid-induced HCO_3^- secretion[23]. The total NO concentration in the EA group was significantly increased with respect to that of the ASA group. This increase of total NO likely protected the gastric mucosa against ASA-induced toxicity. Furthermore, EA-induced NO induction may in turn increase production of PG and HCO_3^-.

In conclusion, EA pretreatment might be effective for gastric defense against ASA-induced ulceration.

COMMENTS

Background

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used as anti-inflammatory and analgesic agents though they often cause gastrointestinal injury in gastric lesions by inhibiting COX. Thus, effective strategies are required to protect the gastrointestinal mucosa.

Research frontiers

In this study, the authors demonstrate that the time-dependent expression of COX could be a potential mechanism for mediating the beneficial effects of acupuncture. The PGs-related acupuncture mechanism should be undertaken, because the endogenous prostaglandins (PGs) are involved in the mucosal defense against chemo-induced ulceration.

Innovations and breakthroughs

The inducible expression of COX-1 in the early phase and COX-2 in the late phase suggests that EA pretreatment might up-regulate the mucosal defense mechanism. The total NO concentration in the EA group was significantly increased with respect to that of the ASA group. The total NO likely protected the gastric mucosa against ASA-induced toxicity. The EA pretreatment might be effective for gastric defense against ASA-induced ulceration.

Application

By understanding the time-dependent expression of COX by EA pretreatment, this study may represent a future strategy for therapeutic intervention in the treatment of patients with gastritis by Nonsteroidal anti-inflammatory drugs.

Terminology

Acupuncture is a technique of inserting and manipulating fine filiform needles into specific points on the body with the aim of relieving pain and for therapeutic purposes. According to traditional Chinese medical theory, these acupuncture points lie along meridians along which qi, the vital energy, flows.

Peer review

The methodology employed is sound and reproducible. The scientific and innovative contents of this manuscript can reflect the advanced levels of the clinical and basic researches in gastroenterology both at home and abroad.

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BRIEF ARTICLES

Surgical palliation of unresectable pancreatic head cancer in elderly patients

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Abstract

AIM: To determine if surgical biliary bypass would provide improved quality of residual life and safe palliation in elderly patients with unresectable pancreatic head cancer.

METHODS: Nineteen patients, 65 years of age or older, were managed with surgical biliary bypass (Group A). These patients were compared with 19 patients under 65 years of age who were managed with surgical biliary bypass (Group B). In addition, the results for group A were compared with those obtained from 17 patients, 65 years of age or older (Group C), who received percutaneous transhepatic biliary drainage to evaluate the quality of residual life.

RESULTS: Five patients (26.0%) in Group A had complications, including one intraabdominal abscess, one pulmonary atelectasis, and three wound infections. One death (5.3%) occurred on postoperative day 3. With respect to morbidity, mortality, and postoperative hospitalization, no statistically significant difference was noted between Groups A and B. The number of readmissions and the rate of recurrent jaundice were lower in Group A than in Group C, to a statistically significant degree ($P = 0.019$, $P = 0.029$, respectively). The median hospital-free survival period and the median overall survival were also significantly longer in Group A ($P = 0.001$ and $P < 0.001$, respectively).

CONCLUSION: Surgical palliation does not increase the morbidity or mortality rates, but it does increase the survival rate and improve the quality of life in elderly patients with unresectable pancreatic head cancer.

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Key words: Adenocarcinoma; Elderly; Palliative surgery; Pancreas neoplasms

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INTRODUCTION

Pancreatic adenocarcinoma is the fourth most frequent neoplasm of the gastrointestinal tract in Korea and it has increased in incidence over the past 15 years (data from the 2002 Annual Report of the Korea Central Cancer Registry). Its incidence increases with age: people older than 65 years represent the section of the population with the highest risk of developing pancreatic adenocarcinoma[1]. In the year 2000, the world-wide incidence of pancreatic adenocarcinoma was 216367 cases; 60% of patients were over 65 years of age[2].

The tumor is located in the pancreatic head and uncinate process in 70% of cases[3]. Surgical resection and biliary-enteric reconstruction usually provides adequate biliary drainage, but only 10% to 20% of patients can be treated surgically with an intent to cure[4,5]. Under other circumstances, the only option is appropriate

palliation to relieve jaundice, pruritis, pain, and duodenal obstruction[6,7]. However, there is controversy over how to provide optimal palliative treatment. In the 1980s, because of the high morbidity and mortality of surgical procedures, obstructive jaundice was mainly managed by endoscopic biliary stenting or percutaneous transhepatic biliary drainage (PTBD). However, more recent studies show a significant decrease in the perioperative mortality and morbidity associated with bypass surgery[8,9]. Therefore, the surgeons' dilemma of "non-surgical or surgical palliation" remains, especially in treating elderly patients.

We performed a retrospective study to determine if surgical biliary bypass would provide improved quality of residual life and safe palliation in elderly patients with unresectable pancreatic head cancer.

MATERIALS AND METHODS

We reviewed the clinical records of 55 patients with unresectable pancreatic head cancer who underwent palliative treatment at our hospital between January, 2001 and January, 2006. Patients excluded from the study comprised those who had already undergone palliative resection, had already developed gastric outlet obstruction, had no obstructive jaundice at the time of diagnosis, or had histology other than adenocarcinoma. There were 19 patients, 65 years of age or older, who were managed with surgical biliary bypass (Group A), these patients were compared with 19 patients under the age of 65 who were managed using surgical biliary bypass (Group B), to determine if surgical biliary bypass could be performed safely in elderly patients. In addition, the results of patients in Group A were also compared with those obtained from 17 patients, aged 65 or older (Group C), who received only PTBD with or without stenting as definitive palliation for unresectable pancreatic head cancer. The purpose of this comparison was to evaluate the quality of residual life in the two groups, and the following parameters were considered: recurrence of jaundice, gastric outlet obstruction, the number of readmissions, median survival, and median hospital-free survival. Hospital-free survival, in which patients are able to spend their daily life at home or away from the hospital until the final readmission, may be related to quality of life. Diagnostic methods included contrast-enhanced computed tomography, ultrasonography, and magnetic resonance imaging. All patients underwent endoscopy to rule out gastroduodenal obstruction. Contraindication to palliative surgery included the presence of distant metastases on preoperative assessment, concomitant risk factors, or patients' choice. Demogaraphics, perioperative and postoperative data were collected from hospital records and analyzed.

All patients underwent diagnostic or therapeutic PTBD before bypass surgery, and operations were performed without delay to lower the bilirubin level. Surgical biliary bypass included cholecystectomy and biliary-enteric bypass (hepaticojejunostomy or choledochojejunostomy). If the blood supply to the proximal bile duct was not sufficient, hepaticojejunostomy was performed. Gastrojejunostomy was performed in patients at high risk for duodenal obstruction during the survival period (such as in cases with duodenal invasion). The previously inserted PTBD catheter was used for postoperative external biliary drainage. It was removed when the biliary-enteric bypass healed without bile leakage.

PTBD was performed by a radiologist with experience in interventional biliary procedures. Ultrasound was used to locate and access the ducts. A 21-guage Chiba needle was inserted percutaneously under local anesthesia with 1% lidocaine, and advanced horizontally into the right or left intrahepatic duct. After placement of a guide wire across the obstructing lesion, sequential dilation of the track was performed. An 8.5F pigtail catheter was positioned with its tip in the distal bile duct. If the catheter was not manipulated past the obstruction, the catheter was left above the obstruction and placed for external drainage. One to two weeks later, biliary stenting was performed by using a plastic stent (BONASTENT^{TM} Biliary; Standard Sci-Tech Inc., Seoul, Korea), if possible, to allow for internal drainage in patients who did not have biliary bypass surgery. Cholangiography was performed immediately after placement of the catheter to confirm that the pigtail catheter was in the correct position within the bile duct.

Statistics were performed using the SPSS for Windows Statistical Software Package (Version 15.0, SPSS Inc. Chicago, IL, USA). All results are presented as mean ± SD or as median with range. Inter-group differences were compared using the χ^2 test and Fisher's exact test, and differences between means were compared using the Mann-Whitney *U* test. Two-tailed *P* values less than 0.05 were considered statistically significant.

RESULTS

Surgical palliation: Old versus young

Sex ratio, surgical risks as assessed according to the American Society of Anesthesiologists (ASA), and TNM staging according to the American Joint Committee on Cancer (AJCC) 2002 classification were similar between the two groups, A and B. One patient in Group A and 4 in Group B underwent biliary bypass only. The mean preoperative bilirubin level was higher in Group B (11.2 ± 7.9 *vs* 14.5 ± 6.5 mg/dL), but not to a statistically significant degree (Table 1). The mean minimal postoperative bilirubin level was significantly lower in Group A (0.9 ± 0.5 *vs* 1.5 ± 0.6). Five patients (26.0%) in Group A had complications including one intraabdominal abscess, one pulmonary atelectasis, and 3 wound infections. In Group B, there was one case of pulmonary atelectasis with pleural effusion. The morbidity rate was higher in group A, but not to a statistically significant degree. One death (5.3%) occurred in Group A after 3 d. The patient presented with upper gastrointestinal bleeding after one day and

Table 1 Patient characteristics and clinical outcomes of Groups A, B and C

	Group A	Group B	Group C
Patient characteristics			
Number of patients	19	19	17
Mean age	71.6 ± 4.8	55.6 ± 6.8	74.4 ± 5.8
Male/Female	7/12	13/6	11/6
ASA			
Ⅰ	6	8	5
Ⅱ	9	8	9
Ⅲ	4	3	3
Stage			
Ⅲ	16	13	16
Ⅳ	3	6	1
Biliary and gastric bypass	18	15	14.5 ± 6.5
Mean preoperative bilirubin level (mg/dL)	11.2 ± 7.9	14.5 ± 6.5	
Clinical outcomes			2.2 ± 2.7
Mean postoperative bilirubin level (mg/dL)	0.9 ± 0.5	1.5 ± 0.6[b]	1 (5.9%)
Early complications	5 (26%)	1 (5.3%)	1 (5.9%)
30 d mortality	1 (5.3%)	0 (0.0%)	4 (26.7%)[a]
Recurrent jaundice	0 (0.0%)	2 (10.5%)	2 (11.8%)
Gastric outlet obstruction	0 (0.0%)	1 (5.3%)	14 (range, 2-43)[a]
Median postoperative hospital stay (days)	19 (range, 3-73)	20 (range, 13-70)	20[a]
Number of readmissions	9	22	120 (range, 0-230)[b]
Median survival (days)	290 (range, 3-723)	213 (range, 70-510)	150 (range, 2-240)[b]

ASA: American Society of Anesthesiologists. [a]$P < 0.05$, [b]$P < 0.01$ *vs* Group A.

underwent reoperation, but died of multiple organ failure. There were no cases of recurrent jaundice or gastric obstruction in Group A, but 7 patients required 9 readmissions due to fever, pain, poor oral intake, or other reasons. In group B, recurrent jaundice developed in 2 patients (10.5%), gastric outlet obstruction developed in one patient (5.3%). Six patients required 22 readmissions. Median postoperative hospitalization was shorter in Group A (19 d, range 3-73 d), but there was no statistically significant difference between the two groups. Median overall survival in the two groups was not significantly different (Table 1).

Surgical palliation versus non-surgical palliation in old age

Seventeen patients 65 years of age or older underwent PTBD for palliation of pancreatic head cancer. The radiologic findings in these patients included tumor invasion of the superior mesenteric vein or portal vein in 3 patients, superior mesenteric artery in 5 patients, both in 7 patients, hepatic artery in one patient, and hepatic metastases in one patient. Patient age, sex ratio, surgical risk (ASA), and TNM staging were similar between the two groups. The mean preoperative bilirubin level was higher in Group C (11.2 ± 7.9 *vs* 14.5 ± 8.7 mg/dL), but not to a statistically significant degree (Table 1). In Group A, successful biliary drainage was observed in 18 of 19 patients (94.7%). Successful biliary drainage was observed in 15 of 17 patients (88.2%) in Group C. There was no statistically significant difference between the two groups. Successful bile drainage was defined as a reduction in the bilirubin level (total bilirubin < 2.0 mg/dL) and improvement in symptoms. The difference in morbidity and mortality rates were not statistically significant between the two groups. One of the 17 patients in Group C (5.9%) had an early complication due to dislodgment of the catheter. One death (5.9%) occurred in Group C after 2 d because of massive hemobilia. Nine patients in Group C required 20 readmissions due to cholangitis, recurrent jaundice, pain, or other reasons. Recurrent jaundice developed in 4 patients (26.7%), and gastric outlet obstruction developed in 2 patients (11.8%). Three of these patients underwent reinsertion of the PTBD, one patient underwent endoscopic endoprosthesis, and 2 patients underwent duodenal stent placement. The number of readmissions, and rate of recurrent jaundice were higher in Group C to a statistically significant degree ($P = 0.019$, $P = 0.029$, respectively). Median postoperative hospitalization was shorter in Group C (14 d, range 2-43 d). The median hospital-free survival period was significantly longer ($P = 0.001$), and median overall survival was significantly longer ($P < 0.001$) in Group A compared to Group C.

DISCUSSION

The risk of developing pancreatic cancer dramatically increases with age, with a median age of 72 years at the time of diagnosis[10]. Thus, the epidemiology of the disease combined with the growth of the elderly population is leading to an increasing number of cases. Even if most patients with pancreatic head cancer are not candidates for radical surgical resection, because of early metastatic spread or extensive local tumor involvement, palliation of obstructive symptoms and pain remains a core component in the management of this disease. Di Carlo *et al*[11] reported there was no significant difference in the frequency of locally advanced or metastatic disease

in elderly patients (over 70 years) compared with those under 70 years. However, to our knowledge there are few reports on the use of surgical biliary bypass to manage unresectable pancreatic head cancer in old age[12,13]. Although endoscopic stenting of the bile duct or PTBD can relieve biliary obstruction, surgical bypass is done in many cases because of patient or physician preference, an inability to access the bile duct, or failure of non-surgical palliation[14]. Surgical bypass can also be performed when a pancreatic head cancer proves to be unresectable during an operation intended to cure the tumor. Significant advances have been made in non-operative palliation for periampullary cancer. Percutaneous or endoscopic palliation of obstructive jaundice can provide biliary decompression with lower early morbidity compared to open biliary bypass surgery[15-18]. However, these techniques have had disappointing outcomes with regard to recurrent jaundice. Several studies have compared PTBD with endoscopic endoprosthesis in malignant biliary obstruction. The endoscopic approach proved to be safer and more effective compared with PTBD[19,20]. However, the long-term complications of both these procedures make them less desirable than surgical bypass in those patients who are expected to survive more than a few months[21].

It may be a common conception that elderly patients are more susceptible to an increased mortality, morbidity and longer hospitalization than their younger counterparts. Interestingly, reluctance to advise an operation is often unrelated to the presence of co-morbidities or impaired functional status[22].

In recent studies, the morbidity of palliative double bypass surgery (biliary-enteric reconstruction, gastrojejunostomy) has ranged between 4.8% and 28%, and mortality has ranged between 1% and 9%[6,7,23,24]. Nuzzo *et al*[12] reported that the morbidity and mortality rates for surgical palliation in elderly patients (> 70 years) with periampullary cancer were comparable to those of younger patients (≤ 70 years), with no statistically significant difference found between the 2 groups. In our study, postoperative morbidity and mortality rates in elderly patients were 26% (5 patients) and 5.3% (one patient), respectively. These figures are comparable to rates reported in other series. Also, there was no significant difference between elderly patients and younger patients in terms of mortality and morbidity rates. Median postoperative hospitalization was 19 d in elderly patients (range, 3-73 d), and there was no statistically significant difference between elderly patients and younger patients in this regard.

To evaluate the efficacy of palliative bypass surgery for the treatment of unresectable pancreatic head cancer in elderly patients, the results were compared against those obtained from patients 65 years of age or older who received PTBD. More frequent recurrent jaundice, readmission, and shorter hospital-free survival were noted in the non-surgical palliation group. Overall, the quality of life, assessed by relief of biliary obstructive symptoms, the number of readmissions, and hospital-free survival, was better after surgical biliary bypass than non-surgical palliation. Survival was improved after surgical bypass. The reasons behind this are not clear, but factors that may contribute include relief of biliary obstruction, low rate of recurrent jaundice, and prevention of gastric obstruction. These may help to improve both the nutritional state of the patients and their general well-being. Therefore, older age alone should not be a contraindication to surgical palliation of unresectable pancreatic head cancer, although elderly patients may require more intensive postoperative care. However, our results must be interpreted with caution because of the selection bias inherent in this study. Actually, surgical palliation was performed in patients who did not have metastatic disease on the preoperative imaging studies. Also stenosis of the biliary duct might be higher in the non-surgical palliation group. Thus, long-term survival and a good quality of survival could be achieved in patients who underwent surgical palliation.

In conclusion, surgical palliation does not increase the morbidity and mortality rates, but it does increase the survival rate and improve the quality of life in elderly patients with unresectable pancreatic head cancer. Further clinical observations and prospective, controlled studies are needed to elucidate the long-term effects of this procedure.

COMMENTS

Background

Even if most patients with pancreatic head cancer are not candidates for radical surgical resection, because of early metastatic spread or extensive local tumor involvement, palliation of obstructive symptoms and pain remains a core component in the management of this disease. There are few reports on the use of surgical biliary bypass to manage unresectable pancreatic head cancer in old age.

Research frontiers

Significant advances have been made in non-operative palliation for periampullary cancer. Percutaneous or endoscopic palliation of obstructive jaundice can provide biliary decompression with lower early morbidity compared to open biliary bypass surgery. However, these techniques have had disappointing outcomes with regard to recurrent jaundice. We performed a retrospective study to determine if surgical biliary bypass would provide improved quality of residual life and safe palliation in elderly patients with unresectable pancreatic head cancer.

Innovations and breakthroughs

There was no significant difference between elderly patients and younger patients in terms of mortality and morbidity rates. The quality of life, assessed by relief of biliary obstructive symptoms, the number of readmissions, and hospital-free survival, was better after surgical biliary bypass than non-surgical palliation. Survival was improved after surgical bypass.

Applications

Surgical palliation does not increase the morbidity and mortality rates, but it does increase the survival rate, and improve the quality of life in elderly patients with unresectable pancreatic head cancer.

Peer review

The authors found that surgical palliation does not increase the morbidity and mortality rates, but it does increase the survival rate and improve the quality of life in elderly patients with unresectable pancreatic head cancer. This is an important study.

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Genetic diagnosis strategy of hereditary non-polyposis colorectal cancer

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Abstract

AIM: To study the characteristics of mismatch repair gene mutation of Chinese hereditary non-polyposis colorectal cancer (HNPCC) and hMLH1 gene promoter methylation, and to improve the screening strategy and explore the pertinent test methods.

METHODS: A systematic analysis of 30 probands from HNPCC families in the north of China was performed by immunohistochemistry, microsatellite instability (MSI), gene mutation and methylation detection.

RESULTS: High frequency microsatellite instability occurred in 25 probands (83.3%) of HNPCC family. Loss of hMLH1 and hMSH2 protein expression accounted for 88% of all microsatellite instability. Pathogenic mutation occurred in 14 samples and 3 novel mutational sites were discovered. Deletion of exons 1-6, 1-7 and 8 of hMSH2 was detected in 3 samples and no large fragment deletion was found in hMLH1. Of the 30 probands, hMLH1 gene promoter methylation occurred in 3 probands. The rate of gene micromutation detection combined with large fragment deletion detection was 46.7%-56.7%. The rate of the two methods in combination with methylation detection was 63.3%.

CONCLUSION: Scientific and rational detection strategy can improve the detection rate of HNPCC. Based on traditional molecular genetics and combined with epigenetics, multiple detection methods can accurately diagnose HNPCC.

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Key words: Hereditary non-polyposis colorectal cancer; Gene mutation; Mismatch repair; hMSH2; hMLH1; Large fragment deletion; Methylation

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INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) is a dominant autosomal genetic syndrome caused by germ-line mutation of mismatch repair gene[1], accounting for 5%-10% of all colorectal cancer[2,3]. Genetic linkage analysis and genetics show that about 80% of HNPCC are associated with germ-line mutation of hMLH1 and hMSH2[4-6]. Most of these mutations are micromutation (also known as a point mutation), small fragment insertion or deletion, *etc*[7,8]. Large fragment deletion in hMLH1 and hMSH2 (especially hMSH2) gene is another way of germ-line mutation[9]. In addi-

tion, recent epigenetic studies indicate that CpG island methylation in hMLH1 gene promoter region is also a mechanism underlying gene inactivation and tumorigenesis[10]. Therefore, systematic and comprehensive detection analysis of 30 samples from HNPCC families in the north of China was performed using various methods.

MATERIALS AND METHODS

Materials

Between 2000 and 2002, 30 probands from HNPCC families were collected and registered by General Hospital of Beijing Military Command and other hospital in Henan, Hebei and Shandong Provinces, and Inner Mongolia Autonomous Region. All study methods were approved by the Ethics Committee of General Hospital of Beijing Military Commad and the 30 probands from HNPCC families gave written consent to participate in the study. Twenty-one families were in line with Bethesda Guideline (BG) Ⅰ, 7 families Bethesda Guideline Ⅲ and 2 families Bethesda Guideline Ⅳ, respectively[11]. With probands as the core, HNPCC genealogical tree of at least two generations was drawn. Pedigree analysis was performed. Family member files including the age when the tumor was diagnosed, the relation to proband, tumor type and location, *etc* (including parenteral cancer) were established.

Methods

DNA extraction: Tumor and normal tissues were obtained from probands. Tumor tissue was fixed with formalin, embedded in paraffin, cut into 6-μm thick sections which were stained with 0.1% methylthioninium chloride. The location (more than 80% tumor tissue) most suitable to microdissection was marked and microdissection was done. DNA was extracted from tumor and normal tissues with phenol/chloroform/isoamyl alcohol for microsatellite instability (MSI) and immunohistochemistry detection[12]. DNA was extracted from venous blood for gene mutation and methylation detection.

Microsatellite instability and immunohistochemistry detection: Microsatellite instability detection was performed in 5 microsatellite markers: D2S123, D5S346, BAT-25, BAT-26 and BAT-40[13,14]. For hMSH2 and hMLH1, immunohistochemical staining was done with the standard biotin-avidin-peroxidase complex method as previously described[15].

hMSH2 and hMLH1 gene mutation detection: Micromutation detection was performed in 25 samples with high microsatellite instability. PCR was performed to amplify all exons (including intron-exon junction) of hMSH2 and hMLH1. PCR products were sequenced with a DNA automatic sequencer (ABIPRISM 3730XL) to find micromutation in the samples and to determine their mutation type.

Large fragment deletion detection in hMSH2 and hMLH1: Multiplex ligation-dependent probe amplification (MLPA) technique[16] was used to detect large fragment deletion in the samples without micromutation with a hMLH1 and hMSH2 large fragment deletion kit purchased from MRC Holland Company. The major steps of MLPA technique include to probe hybridization with the target sequence and specific ligation, to amplify hMSH2 and hMLH1 by PCR with probes, and to analyze PCR products. The PCR products were applied to a ABIPRISM3730 sequencer containing 6% polyacrylamide gel for electrophoresis. The electrophoresis results were analyzed with GeneMapper 3.0 software. The peak of each exon was compared with that of control sample. If the relative height was reduced by 35%-55%, the fragment was determined to have exon deletion. If the relative height was increased by 30%-55%, the fragment was determined to have exon duplication. If the peak was 0, the fragment was determined to have homozygous deletion.

Methylation detection: hMLH1 gene promoter methylation detection was performed in 30 samples. First, DNA was sulfurized with an EZ DNA methylation-gold kit purchased from ZYMO RESEARCH Company, and then methylation-specific PCR (MSP)[17] was performed. PCR amplification was performed twice for each sample with methylation and non-methylation primers, respectively. PCR products were applied to a 10% non-denaturing polyacrylamide gel for electrophoresis, then stained with ethidium bromide, and observed under an ultraviolet lamp.

RESULTS

Clinical and pathological information and family history

Of the 30 families, 21 were in line with BG Ⅰ, 7 were in line with BG Ⅲ and 2 were in line BG Ⅳ (Table 1). One hundred and forty tumors were found in 106 of the 708 members in these families. Of the 140 tumors, 22 (15.7%, 22/140) were extracolonic cancers. Of the 22 tumors, 7 were gastric cancers which are the most common type of extracolonic cancer. Of the colon cancers, 86 (72.9%, 86/118) were right colon cancers and 32 (27.1%, 32/118) were left colon cancer. One patient had synchronous multiple-primary cancers and 7 had metachronous multiple-primary cancers.

Microsatellite instability analysis and mismatch repair protein expression

Microsatellite instability analysis and mismatch repair protein expression in probands are shown in Table 1. Of the 30 samples detected, high frequency microsatellite instability (MSI-H) occurred in 25 samples (83.3%), low frequency microsatellite instability (MSI-L) in one sample (3.3%) and microsatellite stability (MSS) in 4 samples (13.3%). Of the 5 microsatellite loci, MSI-H expression rate was 100% (25/25) and 96% (24/25) in BAT-25 and BAT-26, respectively. Of the 25 samples with MSI-H, loss of hMLH1 or hMSH2 protein expression occurred in 22 (88%), loss of hMLH1 protein expression occurred in 12, and loss of hMSH2 protein expression in

Table 1 Detection results in 30 probands from HNPCC families

Family	Bethesda guidelines	MSI	Expression of MMR proteins		Micromutation/polymorphism[2]/large fragment deletion		hMLH1 gene promoter methylation
			MSH2	MLH1			
H1	BG1	MSI-H	+	+			u
H4	BG1	MSI-H	-	+	hMSH2 exon13	IVS13-2 A→C (SA of Exon 14)	u
H9	BG1	MSI-H	-	+	hMSH2 exon3	c.610G→T (G204stop)	u
H17	BG1	MSI-H	+	+	hMSH2 exon5	c.899_890insAT1	u
H22	BG1	MSI-H	-	+	hMSH2 exon7	IVS7-1G→A (SA of Exon 8)[1]	u
H10	BG1	MSI-H	+	+	hMSH2 exon15	c.2583A→G (Q861Q)[1,2]	u
H2	BG1	MSI-H	-	+	hMSH2 exon8	deletion	u
H5	BG1	MSI-H	-	+			u
H11	BG3	MSI-H	-	+			u
H23	BG1	MSI-H	-	+	hMSH2 exon1-6	deletion	u
H25	BG3	MSI-H	-	+			u
H13	BG1	MSI-H	-	+	hMSH2 exon 7	c.1231 insertion T shift	u
H34	BG4	MSI-H	-	+	hMSH2 exon1-7	deletion	u
H3	BG1	MSI-H	+	-	hMLH1 exon18	c.2041G→A (A681T)	u
H12	BG3	MSI-H	+	-	hMLH1 exon15	IVS15+1 G→A (SD of Exon 15)	u
H19	BG1	MSI-H	+	-	hMLH1 exon8	c.677G→A (splice site mutation)	u
H20	BG1	MSI-H	+	-	hMLH1 exon8	c.677G→A (splice site mutation)	u
H21	BG1	MSI-H	+	-	hMLH1 exon19	c.2141G→A (W714stop)	u
H28	BG1	MSI-H	+	-	hMLH1 exon8	c.655A→G (I219V)	m
H29	BG1	MSI-H	+	-	hMLH1 exon6	c.503_4insA1	u
H30	BG1	MSI-H	+	-	hMLH1 exon9	IVS9+1 G→A (SD of Exon 9)	u
H14	BG1	MSI-H	+	-			u
H18	BG1	MSI-H	+	-			u
H33	BG3	MSI-H	+	-			u
H35	BG3	MSI-H	+	-	hMLH1 exon17	c.1930 del G	u
H8	BG4	MSI-L	+	+			m
H6	BG3	MSS	+	+			m
H36	BG1	MSS	+	+			u
H15	BG3	MSS	+	+			u
H27	BG1	MSS	+	+			u

+: Expression; -: Deletion; [1]Novel mutation discovered; [2]Polymorphism; m: Methylation; u: Non-methylation; MSI-H: High frequency microsatellite instability; MSI-L: Low frequency microsatellite instability; MSS: Mcrosatellite stability.

10, respectively. No loss of mismatch repair protein expression was found in the other 8 samples.

DNA sequencing

Of the 25 samples with MSI-H, pathogenic mutation (Table 1) was detected in 14 (56%). Of the 14 samples, hMLH1 and hMSH2 gene mutation occurred in 9 and 5, respectively. The detection rate of micromutation was 46.7% (14/30). Three novel mutational sites were discovered. Of the 3 novel mutations, a frame shift mutation at c.503_4insA was located in hMLH1, and another frame shift mutation at c.899_890ins AT and a splicing mutation at IVS7-1G→A, SA of Exon 8 were located in hMSH2. A new base replacement (hMSH2, c.2583A →G) was detected in sample H10, which did not cause changes in amino acid sequence. There was no co-segregation phenomenon between this base replacement and disease in its family, suggesting that it is a new change in polymorphism.

Large fragment deletion detection

Electrophoresis scanning peaks in exons 1-6, 1-7 and 8 of hMSH2 in 3 samples were reduced by over 35% (Figure 1), demonstrating that these exons have deletions which are heterozygotic in nature. Large fragment deletion was not detected in hMLH1. Large fragment deletion in hMSH2 accounted for 37.5% of all hNSH2 pathogenic mutations and 17.6% of total hMLH1 and hMSH2 mutations, respectively. Of the 30 samples, micromutation and large fragment deletion were detected in 17, the detection rate of combined methods was 56.7% (17/30).

Methylation detection

hMLH1 gene promoter methylation occurred in 3 of the 30 samples. The detection rate of combined micromutation, large fragment deletion and methylation detection was 63.3% (19/30). MSP electropherogram (Figure 2) showed that hMLH1 gene promoter methylation occurred in 3 samples. Exhaustive methylation occurred in 2 of the 3 samples with their electropherograms displaying M specific fragment but no U deletion, and partial methylation occurred in one of the 3 samples with its electropherogram displaying M and U fragments. Only U specific fragment occurred in the other samples, indicating that no methylation occurs in these samples.

DISCUSSION

Germ-line mutation of mismatch repair gene is the molecular genetic basis of HNPCC pathogenesis. Mismatch repair gene mutation may lead to truncation

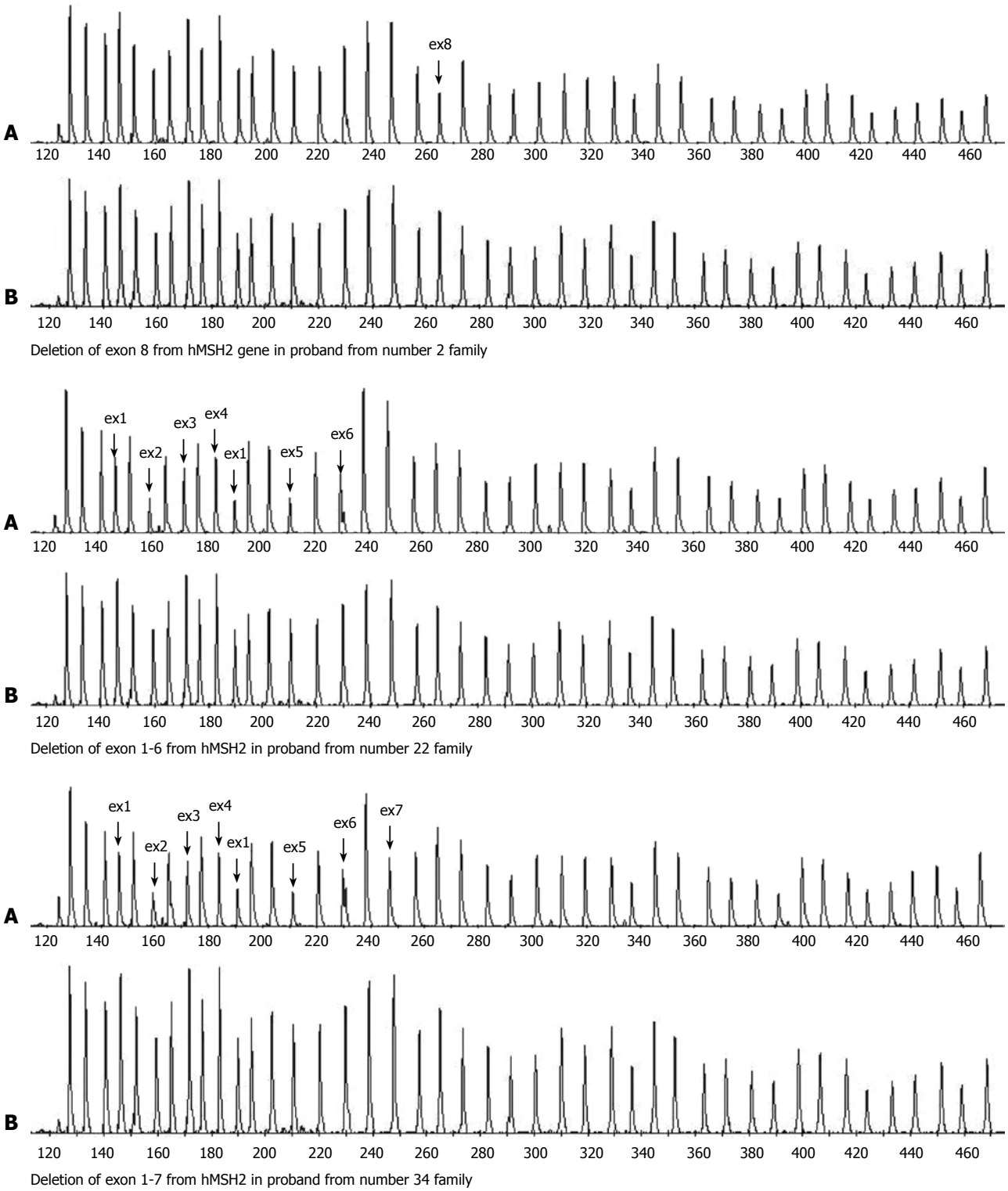

Figure 1 Large fragment deletion results with MLPA technique. A indicates peak graph of fluorescence intensity of proband PCR products. B indicates peak graph of fluorescence intensity of control PCR products. ↓ indicates large fragment deletion in exon.

and lower expression of mismatch repair protein, increasing DNA replication errors and microsatellite instability which results in tumorigenesis[18,19]. Mutations in hMSH2 and hMLH1 are most common[4-6]. Therefore, we mainly studied the two genes.

Microsatellite instability detection was performed in 5 microsatellite markers, including D2S123, D5S346, BAT-25, BAT-26 and BAT-40. The MSI-H detection rate was 83.3%, demonstrating that the incidence of MSI-H is high in patients with HNPCC, and microsatellite instability is one of the most important features of HNPCC and reflects mismatch repair gene state at a certain extent. Loss of mismatch repair protein expression occurred in 88% MSI-H samples while no loss of mismatch repair protein expression occurred in MSI-L and MSS samples, indicating that the specificity, sensitiv-

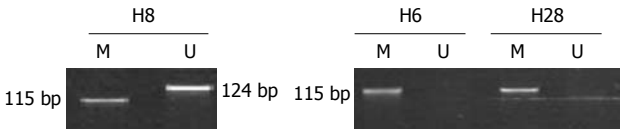

Figure 2 Electropherogram of hMLH1 gene promoter methylation detection. M: Methylation; U: Non-methylation; H: Family number. H8 manifests partial methylation. H6 and H28 manifest exhaustive methylation.

ity and consistency of MSI and immunohistochemistry detection are higher in HNPCC families, the detection methods are simple and economic, and the results of combined detection methods may be used as effective screening indicators before genetic testing.

In MSI-H samples, the detection rate of micromutation in hMSH2 and hMLH1 was 56%. Micromutations of hMLH1 occurred in exons 6, 8, 9, 15, 17-19 and accounted for 64.3%. Micromutations of hMSH2 occurred in exons 3, 5, 7 and 13 and accounted for 35.7%. The mutation types included frame shift, non-sense, splicing and missense mutations. By searching the database of International Society for Gastrointestinal Hereditary Tumors (http://www.insight-group.org), we found 3 new mutational sites including a frame shift mutation at c.503_4insA in hMLH1, and a frame shift mutation at c.899_890ins AT, and a splicing mutation at IVS7-1G→A, SA of exon 8 in hMSH2. Since these mutations could lead to protein product truncation, further study is needed in a larger-scale population to confirm whether such mutations only occur in the north of China. It is evident that the mutation spectrum of HNPCC mismatch repair gene in Chinese is broad and multiple.

Grabowski *et al*[20] reported that large fragment deletion in hMLH1 and hMSH2 accounts for 17% of all pathogenic mutations, which is almost consistent with the results of our study. The three types of large fragment deletion are in line with the findings reported by Nakagawa *et al*[21]. Large fragment deletion commonly occurs in hMSH2, and should be reckoned with in molecular genetics of HNPCC. Therefore, detection of HNPCC molecular genetics should include large fragment deletion detection.

With the development of epigenetics in recent years, DNA methylation has gradually become a new research focus. In human genome, 5' promoter of 50% genes contains a CpG region, also known as CpG island, with its length > 197 bp. CpG island is in a non-methylation state under normal circumstances. CpG island methylation may lead to loss of gene expression and replication errors[22,23]. The promoter methylation of hMLH1 gene is most common in known mismatch repair genes. The detection rate of hMLH1 gene promoter methylation in our study was almost similar to the reported data[24]. Exhaustive and partial methylations were observed in our study, demonstrating that hMLH1 gene promoter methylation may occur in patients with HNPCC, and the methylation level is different in different individuals. A deletion at c.655A→G (1219 V) in hMLH1, loss of hMLH1 protein expression, and hMLH1 gene promoter methylation were found in sample H28, suggesting that further study is needed to explore the protein expression and regulation of HNPCC pathologic mechanism.

An overview of the whole process of detection analysis of the 30 proband samples, microsatellite instability was first performed, then gene micromutation, large fragment deletion and promoter methylation were detected, respectively. The detection rate of gene micromutation was 46.7% (14/30), the detection rate of large fragment deletion detection in combination was increased to 56.7% (17/30). However, the detection rate of three methods in combination was 63.3% (19/30). Almost no comprehensive and systemic detection has been reported both at home and abroad. The detection rate in our study was higher than or similar to reported data[25-27]. Multiple methods in combination may improve the detection efficiency and accuracy of HNPCC and can determine HNPCC families. In order to make early diagnosis and treatment, HNPCC family members should regularly by examined. At the same time, since gene detection is time-consuming and expensive, the cost of various tests and clinical significance should be taken into account according to the actual situation. Therefore, the detection strategy should be made in the following steps. First, families meeting the HNPCC criteria are selected, and then immunohistochemistry and microsatellite instability detection of hMLH1 and hMSH2 are performed. If both of the two detections are negative, mutation detection need not be done. If one of the two detections is positive, micromutations in hMLH1 and hMSH2 should be detected. If micromutation is not detectable, large fragment deletion detection should be considered. MLPA technique can be used in detecting large fragment deletion and is characterized by DNA probe hybridization. PCR technique is rapid, sensitive, specific, reliable and cheap. All the 35 exons of hMLH1 and hMSH2 gene can be detected in the same reaction system with MLPA technique[28]. Epigenetics provides a new idea for the early diagnosis, treatment and prognosis of tumors. Studies indicate that CpG island methylation in the hMLH1 gene promoter region is also a mechanism underlying gene inactivation and tumorigenesis[29,30]. Therefore, based on traditional micromutation detection, we should further combine large fragment deletion detection. For samples without micromutation and large fragment deletion, mismatch repair gene promoter methylation detection should be taken into account. Various detection methods in combination can better diagnose HNPCC.

COMMENTS

Background

Hereditary non-polyposis colorectal cancer (HNPCC), which is caused by a germline mutation in the mismatch repair gene or is associated with tumors exhibiting microsatellite instability (MSI), is characterized by increased risk of developing colon cancer and other cancers, such as cancers of endometrium, ovary, stomach, small intestine, hepatobiliary tract, upper urinary tract, brain, and skin. The diagnosis of HNPCC can be made based on the Amsterdam Clinical Criteria or on molecular genetic testing for germline mutations in one of the mismatch repair (MMR) genes.

Research frontiers

About 80%of HNPCC are associated with germline mutations in hMLH1 and hMSH2. Most of these mutations are micromutation that is a point mutation, small fragment insertion or deletion, *etc*. Large fragment deletion in hMLH1 and hMSH2 gene is another way of germline mutation. In addition, recent epigenetic researches indicate that CpG island methylation in the hMLH1 gene promoter region is also a mechanism underlying gene inactivation and tumorigenesis.

Innovations and breakthroughs

They used combined MSI and immunohistochemistry detection, gene mutation detection (i.e. sequencing and large fragment deletion detection) and methylation detection to study the characteristics of MMR gene mutation and hMLH1 gene promoter methylation in Chinese HNPCC patients. The results demonstrate that the combined MSI and immunohistochemistry detection might be used as effective screening indicators before genetic testing. Furthermore, detection of HNPCC molecular genetics should include large fragment deletion detection. For samples without micromutation and large fragment deletion, mismatch repair gene promoter methylation detection should be taken into account.

Applications

According to their results, the detection strategy should be made in the following steps. First, families meeting the HNPCC criteria are selected, and then immunohistochemistry and microsatellite instability detection of hMLH1 and hMSH2 are performed. If both of the two detections are negative, mutation detection needs not be done. If one of the two detections is positive, micromutations in hMLH1 and hMSH2 should be detected. If micromutation is not detectable, large fragment deletion detection should be considered. For samples without micromutation and large fragment deletion, mismatch repair gene promoter methylation detection should be taken into consideration. Various detection methods in combination may better diagnose HNPCC.

Peer review

The authors performed a systematic analysis of various genetic diagnostic methods in Chinese HNPCC patients and assessed their efficiency. The results are interesting and suggest that MSI, immunohistochemistry detection, gene mutation detection and methylation detection in combination may better diagnose HNPCC.

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BRIEF ARTICLES

Comparative study of omeprazole, lansoprazole, pantoprazole and esomeprazole for symptom relief in patients with reflux esophagitis

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Abstract

AIM: To clarify whether there is any difference in the symptom relief in patients with reflux esophagitis following the administration of four Proton pump inhibitors (PPIs).

METHODS: Two hundred and seventy-four patients with erosive reflux esophagitis were randomized to receive 8 wk of 20 mg omeprazole (n = 68), 30 mg of lansoprazole (n = 69), 40 mg of pantoprazole (n = 69), 40 mg of esomeprazole (n = 68) once a day in the morning. Daily changes in heartburn and acid reflux symptoms in the first 7 d of administration were assessed using a six-point scale (0: none; 1: mild; 2: mild-moderate; 3: moderate; 4: moderate-severe; 5: severe).

RESULTS: The mean heartburn score in patients treated with esomeprazole more rapidly decreased than those receiving other PPIs. Complete resolution of heartburn was also more rapid in patients treated with esomeprazole for 5 d compared with omeprazole (P = 0.0018, P = 0.0098, P = 0.0027, P = 0.0137, P = 0.0069, respectively), lansoprazole (P = 0.0020, P = 0.0046, P = 0.0037, P = 0.0016, P = 0.0076, respectively), and pantoprazole (P = 0.0006, P = 0.0005, P = 0.0009, P = 0.0031, P = 0.0119, respectively). There were no significant differences between the four groups in the rate of endoscopic healing of reflux esophagitis at week 8.

CONCLUSION: Esomeprazole may be more effective than omeprazole, lansoprazole, and pantoprazole for the rapid relief of heartburn symptoms and acid reflux symptoms in patients with reflux esophagitis.

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Key words: Omeprazole; Pantoprazole; Lansoprazole; Esomeprazole; Reflux esophagitis; Symptom relief

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Zheng RN. Comparative study of omeprazole, lansoprazole, pantoprazole and esomeprazole for symptom relief in patients with reflux esophagitis. *World J Gastroenterol* 2009; 15(8): 990-995 Available from: URL: http://www.wjgnet.com/1007-9327/15/990.asp DOI: http://dx.doi.org/10.3748/wjg.15.990

INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common disorder with a high incidence rate of 10%-38% in the Western population occurring at least once a week[1,2]. The prevalence of GERD has been increasing[3].

The severity of GERD is directly correlated with the degree and duration of esophageal acid exposure and is highly pH dependent[4-6]. Chronic exposure is associated with serious complications including esophageal stricture in 4%-20% of patients[4] and Barrett's esophagus in up to 15% of patients with GERD[4-7].

Healing of reflux esophagitis is directly correlated with the intragastric pH > 4.0[8,9]. The efficacy of antisecretory drugs in healing reflux esophagitis depends on the strength and duration of acid suppression within a 24 h period, and the duration of the treatment[10]. Proton pump inhibitor (PPI) therapy is effective for acid-related symptoms. A number of investigators have reported that earlier symptom relief and higher endoscopic healing rates have been obtained with PPI in comparison with H_2-receptor antagonists (H_2-RAs)[11,12]. The time period required to obtain maximal inhibition of gastric acid secretion is, however, reported to differ between PPI[13-17]. The time taken for the resolution of symptoms in patients with reflux esophagitis is, therefore, unlikely to be uniform in all PPI. As the quality of life (QOL) of patients with reflux esophagitis is decreased by heartburn symptoms[18,19], quick symptom relief is important to normalize their QOL. It has not hitherto been fully determined whether differences in the onset of anti-secretary activity may affect the speed

of symptom relief with different PPI. In this study, we investigated the differences in symptom relief in the first 7 d of administration of omeprazole, lansoprazole, pantoprazole, and esomeprazole in patients with reflux esophagitis.

MATERIALS AND METHODS

Two hundred and seventy-four patients with endoscopically proven reflux esophagitis in the Affiliated Hospital of Yanbian University from January, 2006 to September, 2007 and the Affiliated Hospital of Hainan Medical College from October, 2007 to November, 2008 were included in the study. Ten of the patients were lost to follow-up, who refused endoscopic examination after administration of PPI. Subjects with active peptic ulcer, upper gastrointestinal cancers, malignant diseases of other organs, severe cardiac, hepatic, or renal diseases, anemia (hemoglobin concentration < 10 g/dL), or who were pregnant and/or lactating, were excluded. After written informed consent for enrollment in this study was obtained, one of four PPI (omeprazole, lansoprazole, pantoprazole, or esomeprazole, which were contained in sealed envelopes) was administrated for 8 wk (Figure 1). The sealed envelopes containing one of four PPI were randomly assigned for administration. Each PPI was administered once in the morning, 20 mg omeprazole, 40 mg pantoprazole, 30 mg lansoprazole, and 40 mg esomeprazole. Subjects were not permitted to take H_2-RAs or prokinetic drugs during the study period. There were 135 men and 139 women (mean age 57.8 ± 13.5 years, range 36-85 years).

All endoscopic examinations were performed by one endoscopist, using a high-resolution upper gastrointestinal endoscope (GIF 260 series; Olympus, Tokyo, Japan) before and 8 wk after the administration of PPI. Endoscopic diagnosis and the grading of reflux esophagitis were based on the Los Angeles (LA) classification[20]. *Helicobacter pylori* (*H pylori*) infection status was also tested by measuring serum anti-*H pylori* immunoglobulin IgG antibodies, using an ELISA test (Institute of Immunology, Tokyo, Japan).

All patients were asked to keep a symptom diary, in which they recorded the severity of symptoms (heartburn and acid reflux) prior to and during the first 7 d of PPI administration. The severity of symptoms was graded on a six-point scale (0: none; 1: mild; 2: mild-moderate; 3: moderate; 4: moderate-severe; 5: severe and/or intolerable) and was recorded daily. Mild symptoms were defined as a heartburn/acid reflux that did not disturb the normal daily activity of the patients. Moderate symptoms were defined as those that bothered the daily activity, while the patients continued to work productively. Severe symptoms were defined as high-grade symptoms that stopped the daily productive activity of the patients. The patients were instructed to record the severity of their symptoms as a whole previous day's score in the following morning. The daily changes in the severity of two symptoms (heartburn and acid reflux) were separately analyzed. The primary endpoint of the present study was to clarify whether rapid symptom relief in the first week of drug administration differed between the four kinds of PPI.

Statistical analysis

Statistical analysis of inter-group data was performed using the Microsoft Office Excel *F*-test. The Microsoft Office Excel *F*-test was also used to compare the complete disappearance of symptoms between the groups. In addition, sex, age, *H pylori* status and grading of endoscopic esophagitis were analyzed by χ^2 test.

RESULTS

There were no significant differences between the groups in sex, age, *H pylori* status, the grade of reflux esophagitis, and the proportion of cases with heartburn and acid reflux symptoms before the administration of the drugs (Table 1).

No severe side effects related to PPI administration were reported in subjects participating in the present study. None of the patients needed to take antacids for the relief of symptoms after PPI administration.

Figures 2 and 3 show the daily changes in the mean symptom scores of heartburn and acid reflux in all patients with each PPI. Although there were no differences between the groups in the heartburn score before PPI administration, the heartburn score was significantly lower in subjects administered esomeprazole after the first and second days than in those administered omeprazole ($P = 0.0031$, $P = 0.0092$), lansoprazole ($P = 0.0039$, $P = 0.0088$), and pantoprazole ($P = 0.0009$, $P = 0.0036$), respectively. This difference between subjects administered esomeprazole and the other PPI tended to disappear after 5 d of administration of the test drugs. No significant differences in acid reflux scores were seen between the groups (Figure 3).

When the analysis was limited to only the patients who initially reported heartburn and acid reflux, the heartburn score of those subjects administered esomeprazole decreased more quickly than those administered omeprazole, lansoprazole, and pantoprazole (Figure 4). This difference also tended to disappear after 5 d of administration of the test drugs. Complete disappearance of heartburn symptoms after administration of the test drugs from 1 d to 5 d occurred more rapidly in subjects administered esomeprazole than in those administered omeprazole ($P = 0.0018$, $P = 0.0098$, $P = 0.0027$, $P = 0.0137$, $P = 0.0069$, respectively), pantoprazole ($P = 0.0006$, $P = 0.0005$, $P = 0.0009$, $P = 0.0031$, $P = 0.0119$, respectively), and lansoprazole ($P = 0.0020$, $P = 0.0046$, $P = 0.0037$, $P = 0.0016$, $P = 0.0076$, respectively). No significant differences in acid reflux scores were seen between the groups (Figure 5).

Ten of the 274 subjects enrolled in the present study refused endoscopic examination after administration of PPI, so upper gastrointestinal endoscopy was performed in 264 patients at week 8 after the commencement of

Table 1 Characteristics of subjects

	Omeprazole (n = 68)	Lansoprazole (n = 69)	pantoprazole (n = 69)	Esomeprazole (n = 68)	Statistical difference
Sex (male/female)	33/35	35/34	34/35	33/35	NS
Age (mean ± SD) (yr)	57.9 ± 14.1	58.1 ± 13.0	57.8 ± 13.2	57.4 ± 12.8	NS
H pylori (positive/negative)	29/39	31/38	30/39	29/39	NS
Endoscopic esophagitis (Los Angeles classification)					
A	20	20	20	20	NS
B	26	26	28	26	NS
C	20	21	20	20	NS
D	2	2	1	2	NS
Symptoms					
Heartburn (%)	61 (89.7)	63 (91.3)	62 (89.9)	63 (92.6)	NS
Acid reflux (%)	33 (48.5)	35 (50.7)	34 (49.3)	35 (51.5)	NS
No symptom (%)	8 (11.8)	5 (7.2)	8 (11.5)	5 (7.4)	NS

NS: Not significant.

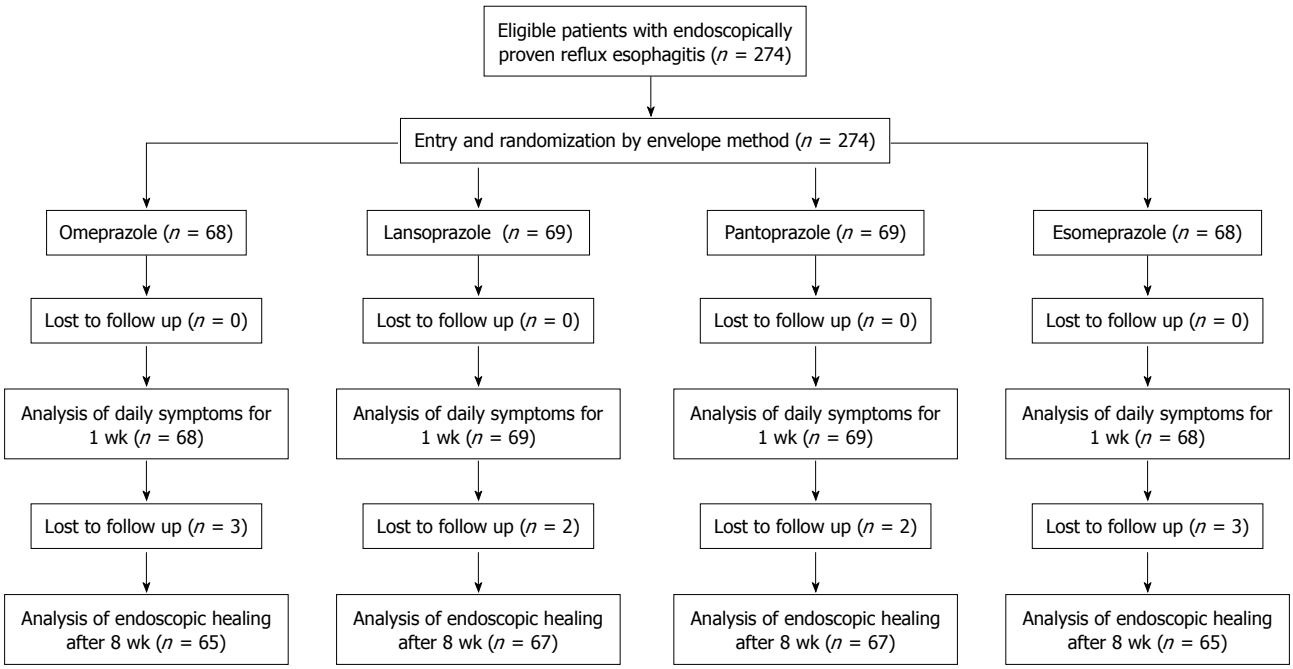

Figure 1 Study protocol.

drugs. These patients had taken all of the PPI. The endoscopic healing rates for reflux esophagitis in subjects administered omeprazole, lansoprazole, pantoprazole and esomeprazole were 87.7%, 89.6%, 91.1% and 95.4%, respectively. Although the healing rate in subjects administered esomeprazole tended to be higher than in those administered omeprazole, lansoprazole and pantoprazole, the differences did not reach statistically significant levels.

When the patients were divided into *H pylori* positive and negative groups, the healing rate for reflux esophagitis at week 8 in *H pylori* positive patients tended to be higher than that in negative subjects (92.4% *vs* 85.8%, $P > 0.05$, $\chi^2 = 2.95$, by χ^2 test).

The daily changes in heartburn score during the first week of administration of the test drugs did not differ between *H pylori*-positive and *H pylori*-negative patients by *F*-test (Figure 6). There was no significant difference in the complete disappearance of heartburn between *H pylori*-positive and *H pylori*-negative patients (Figure 7).

The daily changes in the acid reflux score during the first week of administration of the test drugs also did not differ between *H pylori*-positive and *H pylori*-negative patients (data not shown).

DISCUSSION

Gastroesophageal reflux disease (GERD) is caused by acid reflux, which can be treated by suppressing gastric acid secretion[21,22]. The efficacy of antisecretory drugs in healing reflux esophagitis depends on the potency of acid suppression[12], and PPIs are considered to be the most effective drugs for reflux esophagitis[23]. The symptoms of reflux esophagitis, such as heartburn, have been demonstrated to markedly impair QOL in these patients[18,19]. Complete and rapid relief of symptoms

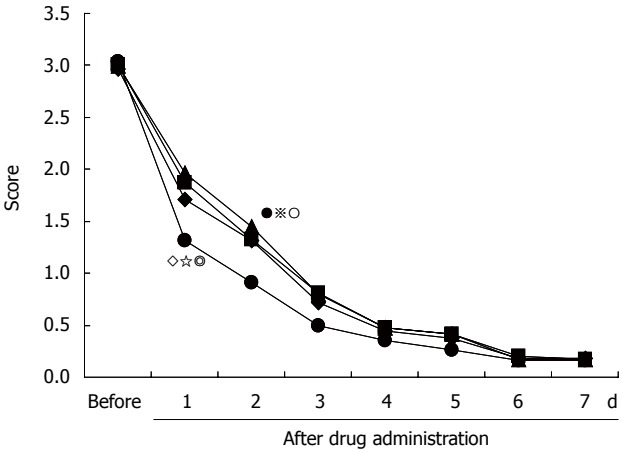

Figure 2 Daily changes in mean heartburn score for all subjects on each proton pump inhibitor regimen. (◆) Subjects administered omeprazole (n = 68), (■) Subjects administered lansoprazole (n = 69), (▲) Subjects administered pantoprazole (n = 69), (●) Subjects administered esomeprazole (n = 68). ◇☆◎ Significant difference between the omeprazole, lansoprazole, pantoprazole, and esomeprazole after 1 d drug administration (P = 0.0031, 0.0039, 0.0009, respectively). ●※○ significant difference between the omeprazole, lansoprazole, pantoprazole and esomeprazole after 2 d drug administration (P = 0.0092, 0.0088, 0.0036, respectively).

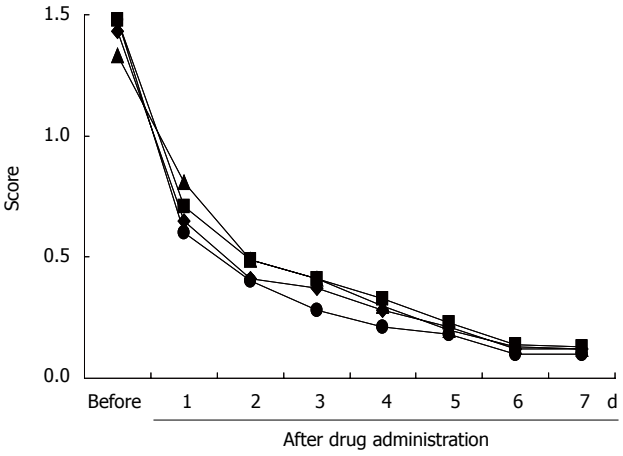

Figure 3 Daily changes in mean acid regurgitation score for all subjects on each proton pump inhibitor regimen. There were no significant differences between the (◆) omeprazole (n = 68), (■) lansoprazole (n = 69), (▲) pantoprazole (n = 69), and (●) esomeprazole (n = 68) groups after drug administration.

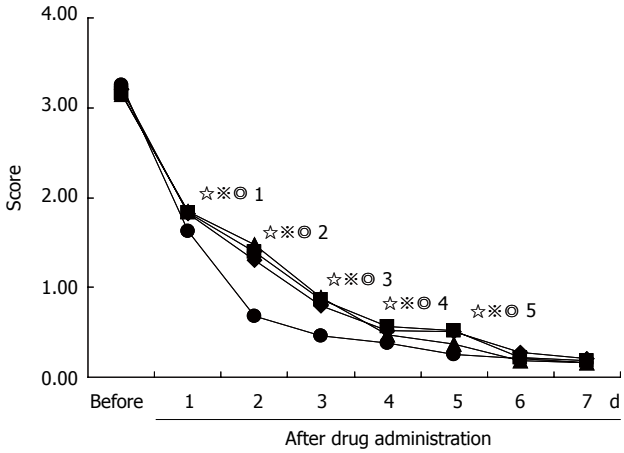

Figure 4 Daily changes in mean heartburn score for all subjects on each proton pump inhibitor regimen, considering only subjects with symptoms prior to commencement of test-drug administration. (◆) Subjects administered omeprazole (n = 61), (■) Subjects administered lansoprazole (n = 63), (▲) Subjects administered pantoprazole (n = 62), (●) Subjects administered esomeprazole (n = 63). ☆※◎△ 1-5 significant difference between the omeprazole, lansoprazole, pantoprazole and esomeprazole after 1-5 d drug administration, P = 0.0018, 0.0020, 0.0006, respectively, P = 0.0098, 0.0046, 0.0005, respectively, P = 0.0027, 0.0037, 0.0009, respectively, P = 0.0137, 0.0016, 0.0031, respectively, P = 0.0069, 0.0076, 0.0119, respectively.

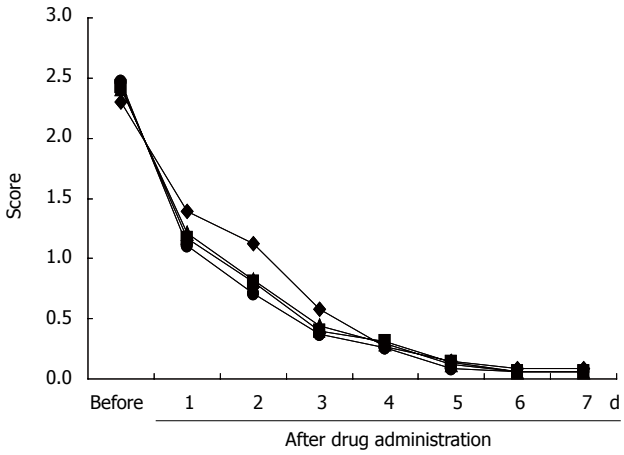

Figure 5 Daily changes in mean acid regurgitation score for considering only subjects with symptoms prior to commencement of test drug administration. There were no significant differences between the (◆) omeprazole (n = 33), (■) lansoprazole (n = 35), (▲) pantoprazole (n = 34), and (●) esomeprazole (n = 35) groups after drug administration.

is, therefore, of critical importance in the treatment of patients with reflux disease.

In the present study, we compared with efficacy of omeprazole, pantoprazole, lansoprazole and esomeprazole for symptom relief in the first 7 d of treatment for reflux esophagitis. The administration of esomeprazole was most effective for symptom relief within 2 d compared with omeprazole, lansoprazole and pantoprazole administration, this difference disappeared 5 d after commencement of drug administration. The results of present study are consistent with those of the study by Rohss *et al*[24-26] and Miner *et al*[27], who reported that esomeprazole 40 mg daily was more effective than omeprazole 20 mg daily, lansoprazole 30 mg, pantoprazole 40 mg daily in the relief of heartburn symptoms during the first day and the first 5 d after the commencement of administration.

In the present study, we demonstrated that esomeprazole gave faster symptom relief than pantoprazole, lansoprazole and omeprazole. Because esomeprazole has been shown to have a faster onset of antisecretory activity than omeprazole, lansoprazole and pantoprazole[24,27], esomeprazole rapidly increased the detectable intragastric pH > 4 on the first treatment day[26], and the first 5 d after commencement of administration of esomeprazole, and the intragastric pH > 4 maintained for a longer period of time than lansoprazole, pantoprazole and omeprazole[25,27].

Although the symptom relief was faster by

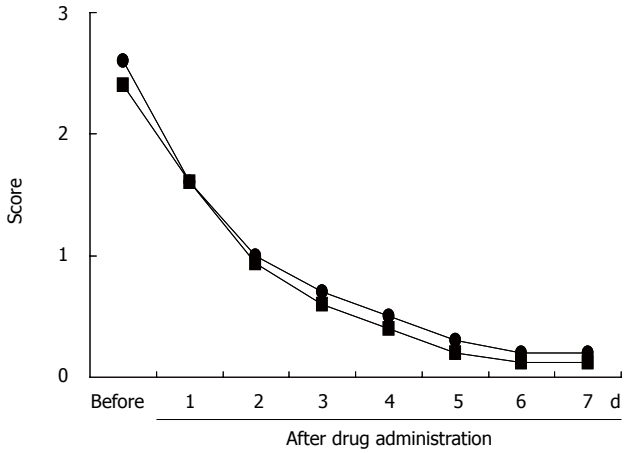

Figure 6 Daily changes in mean heartburn score in *H pylori*-positive and -negative of all subjects with test drug administration. (●) *H pylori*-negative subjects (n = 155), (■) *H pylori*-positive subjects (n = 119), There was no significant difference between *H pylori*-positive and *H pylori*-negative subjects after test drug administration.

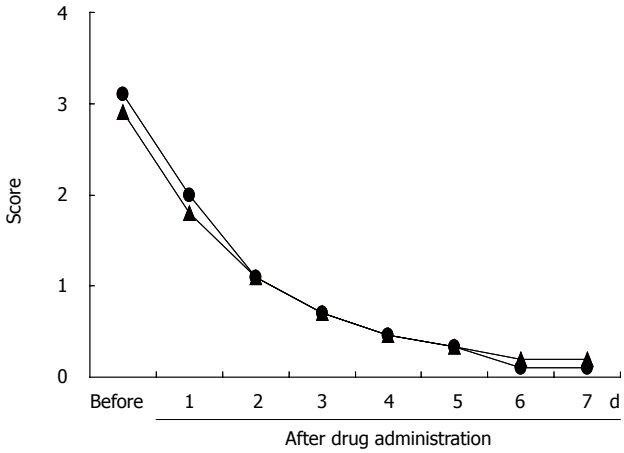

Figure 7 Daily changes in mean heartburn score in *H pylori*-positive and -negative subjects with heartburn prior to test drug administration. (▲) *H pylori*-negative subjects (n = 140), (●) *H pylori*-positive subjects (n = 109), There was no significant difference between *H pylori*-positive and *H pylori*-negative subjects after test drug administration.

administration of esomeprazole than by omeprazole, pantoprazole or lansoprazole, the four kinds of PPI investigated in the present study were demonstrated to be effective for symptom relief within 1 wk in patients with endoscopically proven esophagitis. Recently, PPI has been also used for the diagnosis of gastroesophageal reflux, not only in the patients with non-erosive reflux disease (NERD)[28,29], but also in patients with atypical gastroesophageal reflux symptoms[30,31]. The present study suggests that the four kinds of PPI are effective for the diagnosis of the existence of gastroesophageal acid reflux, and it may be worthwhile investigating whether esomeprazole could shorten the time period necessary for diagnosis.

The healing rate of reflux esophagitis after 8 wk of treatment tended to be higher in patients administered esomeprazole than in those administered omeprazole, lansoprazole or pantoprazole, although these differences did not reach a statistically significant level. However, these differences need to be confirmed by further large comparative studies, which may have been caused by variations in the proportion of *H pylori* positive patients between the four PPI regimen groups, because *H pylori* infection has been reported to influence the healing of reflux esophagitis by PPI[32]. The degree of symptom relief was not, however, different during the first week of administration of PPI between *H pylori* positive and negative patients. The symptomatic response to PPI treatment during the first week administration should not, therefore, be affected by *H pylori* status.

There were several limitations in the comparison of the speed of symptom relief between the four PPIs in the present study since the study subjects were relatively small in number, and some patients with endoscopically proven reflux esophagitis, but without any reflux symptoms were included in the study.

In conclusion, the present study found that esomeprazole 40 mg daily may be more effective than either omeprazole 20 mg daily, pantoprazole 40 mg daily or lansoprazole 30 mg daily for the rapid relief of heartburn symptoms in patients with endoscopically proven reflux esophagitis. Symptom relief after several days of treatment and reflux esophagitis healing rates after 8 wk of treatment, were not different between patients treated with omeprazole, pantoprazole, lansoprazole, or esomeprazole.

COMMENTS

Background

In patients with gastroesophageal reflux disease (GERD), esomeprazole has demonstrated pharmacological and clinical benefits beyond those seen with the other proton pump inhibitors (PPIs). The efficacy of omeprazole, lansoprazole and esomeprazole for symptom relief in the first 7 d of treatment for reflux esophagitis was compared with endoscopic healing rates for reflux esophagitis after 8 wk of treatment.

Innovations and breakthroughs

Although many investigators have reported that esomeprazole can more rapidly increase detectable intragastric pH > 4 on the first treatment day, and the first 5 d than the other PPIs, symptom relief after administration of the lansoprazole, pantoprazole, omeprazole and esomeprazole has not been investigated. This paper showed for the first time that symptom relieved in patients with GERD after administration of the lansoprazole, pantoprazole, omeprazole and esomeprazole. Esomeprazole more rapidly relieved heartburn after administration of the test drugs than the other PPIs.

Peer review

This is a report designed to analyze a symptom relief for GERD subjects based on presenting symptoms and endoscopic healing rate for reflux esophagitis in China. This clinical study was well designed and the manuscript is well written.

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BRIEF ARTICLES

Impact of human immunodeficiency virus infection on the course of hepatitis C virus infection: A meta-analysis

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Abstract

AIM: To analyze the influence of human immunodeficiency virus (HIV) infection on the course of hepatitis C virus (HCV) infection.

METHODS: We performed a meta-analysis to quantify the effect of HIV co-infection on progressive liver disease in patients with HCV infection. Published studies in the English or Chinese-language medical literature involving cohorts of HIV-negative and -positive patients coinfected with HCV were obtained by searching the PUBMED, EMBASE and CBM. Data were extracted independently from relevant studies by 2 investigators and used in a fixed-effect meta analysis to determine the difference in the course of HCV infection in the 2 groups.

RESULTS: Twenty-nine trails involving 16750 patients were identified including the outcome of histological fibrosis or cirrhosis or de-compensated liver disease or hepatocellular carcinoma or death. These studies yielded a combined adjusted odds ratio (OR) of 3.40 [95% confidence interval (CI) = 2.45 and 4.73]. Of note, studies that examined histological fibrosis/cirrhosis, decompensated liver disease, hepatocellular carcinoma or death had a pooled OR of 1.47 (95% CI = 1.27 and 1.70), 5.45 (95% CI = 2.54 and 11.71), 0.76 (95% CI = 0.50 and 1.14), and 3.60 (95% CI = 3.12 and 4.15), respectively.

CONCLUSION: Without highly active antiretroviral therapies (HAART), HIV accelerates HCV disease progression, including death, histological fibrosis/cirrhosis and decompensated liver disease. However, the rate of hepatocellular carcinoma is similar in persons who had HCV infection and were positive for HIV or negative for HIV.

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Key words: Human immunodeficiency virus; Hepatitis C virus; Coinfection; Disease progression; Meta-analysis

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INTRODUCTION

Hepatitis C virus (HCV) infection is a major public health problem, with an estimated global prevalence of 3% occurring in about 170 million infected persons worldwide. The natural history of HCV infection remains controversial because there is a considerable variability in the published estimates of the time span over which cirrhosis develops, as well as the proportion and characteristics of persons in whom it occurs. In common, the natural history of HCV infection remains including acute hepatitis, chronic hepatitis, hepatic cirrhosis, hepatocellular carcinoma, decompensated liver disease and death. Approximately 75%-85% of infected patients do not clear the virus for 6 mo, and chronic hepatitis develops. An estimated 5%-20% of HCV-infected patients have or will develop cirrhosis, and 1%-4% of them will annually develop hepatocellular carcinoma. The disease progress of HCV infection may be affected by many factors, including the age of infection, gender, ethnicity, duration of infection, alcohol consumption, mode of acquisition, and immunosuppression[1].

Coinfection with human immunodeficiency virus (HIV) and HCV, a major public health problem,

frequently shares the blood, sexual, and mother-to-child routes of transmission[2-4]. It is estimated that 4-5 million patients are coinfected with HIV and HCV in the world[5]. The prevalence of HIV-HCV coinfection is even up to 90% in persons injecting drugs[6].

Before an introduction of highly active antiretroviral therapies (HAART), the impact of HCV on the course of HIV infection is overshadowed by extrahepatic cause of death, related to immunodeficiency factors, namely opportunistic infection, lymphomas or wasting syndrome. The development of HAART results in a significant decrease in morbidity and mortality among HIV-infected patients. HCV is the leading non-AIDS cause of death in coinfected persons[7-9]. There is convincing evidence that coinfection with HIV worsens the prognosis of HCV-related liver disease. It was reported that persons coinfected with HIV and HCV would develop cirrhosis, and the incidence of end-stage liver disease is higher in HCV-infected individuals[10,11], especially in individuals with CD4 < 200 cells/μL and alcohol consumption[12]. Graham performed a meta-analysis of eight studies in 2001 to examine the risk of cirrhosis and ESLD in individuals coinfected with HIV and HCV and infected with only HCV, and found that the risk of progressing to cirrhosis and liver failure in individuals coinfected with HIV and HCV is two-fold and six-fold higher, respectively, than in those infected with only HCV[13]. However, this study did not compare the end-point event of death between the two groups. Since then, a large number of cohort studies showing the effect of HIV on liver disease progression have been published. Based on the above data, we conducted a meta-analysis of published studies to investigate the impact of HIV coinfection on the course of HCV, decompensated liver disease, cirrhosis, hepatocellular carcinoma and death.

The aim of this analysis was to summarize the main characteristics of the included studies in order to provide a point estimate of the effect of HIV-HCV coinfection on progressive liver disease compared with HCV infection, to examine the potential heterogeneity, and to identify the potential confounding variables in these studies.

MATERIALS AND METHODS

Literature search

Using variations on the terms of human immunodeficiency virus, AIDS, acquired immunodeficiency syndrome, hepatitis C, cohort study, death, end-stage liver disease, hepatocellular carcinoma, hepatic cirrhosis, and hepatic fibrosis, we conducted a search of the available studies published in English and Chinese from PUBMED, EMBASE and CBM from 1992 when HCV EIA became available to August 30, 2008 to show how concurrent HIV infection changes the course of hepatitis C infection. Combined key words were used to maximize the search results. The bibliographies of selected articles and reviews were also searched for pertinent studies.

Inclusion and exclusion criteria

All identified articles were screened, and we excluded articles that were determined to be irrelevant on the basis of a review of the title and/or abstract. Full texts of all remaining articles were retrieved and reviewed.

Only full-length and peer-reviewed original journal articles were included. Articles that did not provide the number of patients infected with HCV and HIV and clinical outcomes of liver disease were excluded. The remaining articles were independently examined in detail by at least 2 of the observers for the number of patients with HIV-HCV coinfection compared to those with only HCV infection. HCV infection was defined by a positive result of a second or third generation of HCV ELISA and confirmed by recombinant immunoblot assay or PCR. HIV infection was defined by a positive result of HIV ELISA and confirmed by Western blot assay. Patients selected for study did not exclude living patients to avoid bias against non-terminal severe liver disease. Outcomes included histopathological diagnosis of cirrhosis based on the criteria defined by Knodell *et al*[14] or clinically defined decompensated liver disease defined unambiguously as the presence of ⩾ 2 of the following conditions: bleeding esophageal varices, ascites, hepatic encephalopathy, or persistent conjugated hyperbilirubinemia not attributable to medications or hepatocellular carcinoma confirmed by ultrasonic or histopathological diagnosis, or mortality.

Data extraction

Quantitative data on the number of cohort subjects with HCV infection or HCV-HIV coinfection were extracted, and the number of patients in each infection group with the outcome of clinical decompensated liver disease or histological cirrhosis, hepatocellular carcinoma, and death was calculated. Contingency tables were created, and results were converted to odds ratio (OR). When risk estimates were presented, we used those adjusted for the greatest number of potential confounders.

Two independent reviewers abstracted each article separately. Where discrepancies arose, a third investigator arbitrated. When a report ⩾ 1 appeared to describe the same cohort of patients (which was established based on the cohort location and authors involved), we selected the most recent or the most complete study.

Statistical analysis

Analyses were conducted with Review manager (version 4.2, Cochrane Collaboration, Oxford, UK). We assessed the heterogeneity for each pooled estimate with Cochran's Q test. We used a fixed-effect model because of the anticipated variability among trails regarding the different outcomes. The overall mean difference was estimated. The significance was measured at $P < 0.05$. Significant heterogeneity was measured at $P < 0.10$. In the event of significant heterogeneity, results were further analyzed with respect to the outcomes of trails, such as histopathological diagnosis of cirrhosis, decompensated liver disease, hepatocellular carcinoma, and death. Finally, we conducted sensitivity analyses

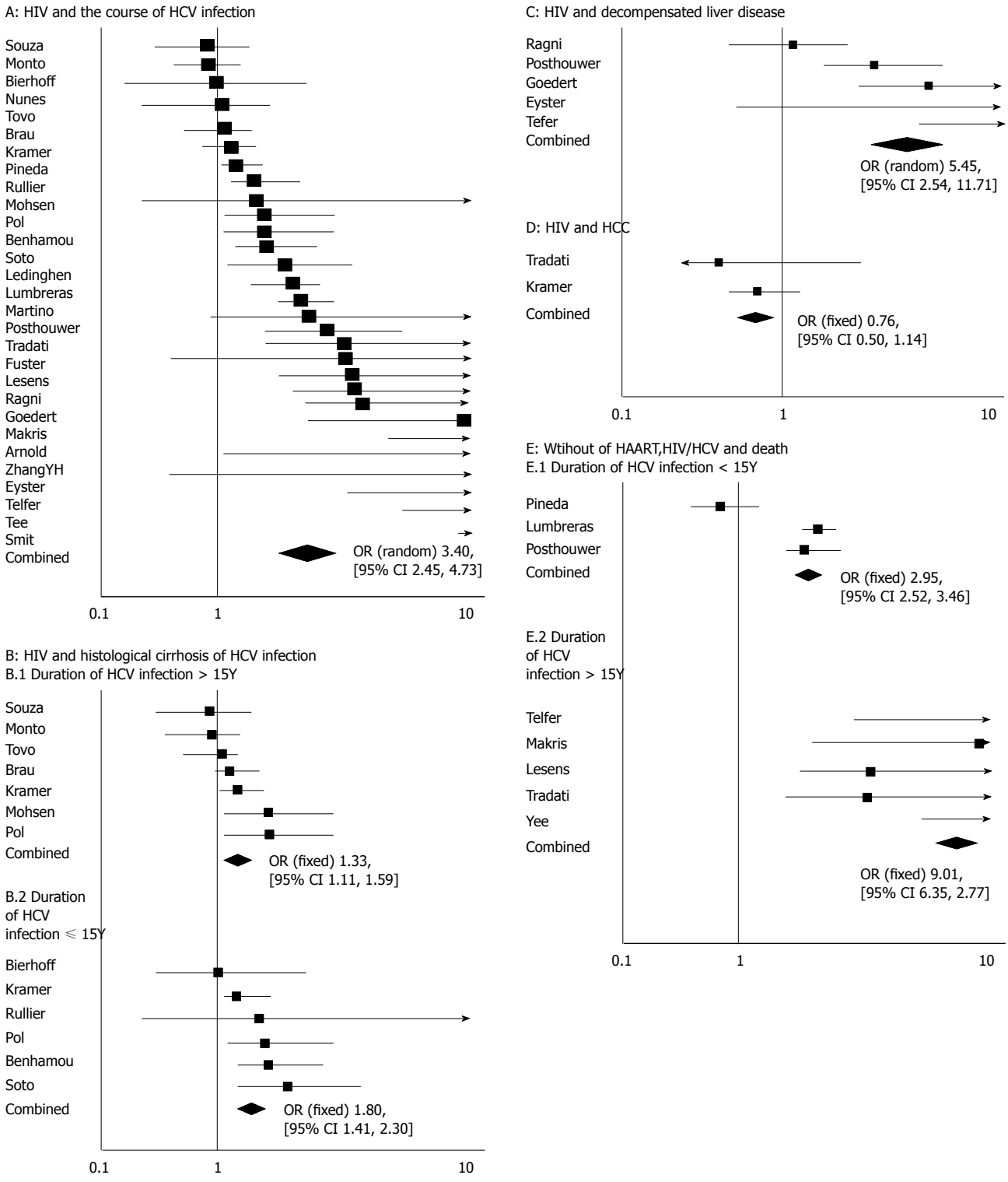

Figure 1 Impact of HIV infection on the course (A), cirrhosis (B), de-compensated liver disease (C), liver cancer (D), and death (E) in HCV-infected patients.

omitting each study in turn to determine whether the results were influenced excessively by a single study.

RESULTS

Included studies

After searching the PUBMED, EMBASE and CBM, a total of 422 studies were identified and screened for retrieval. Two hundred and ninety-seven case reports, case-control studies, or review articles were excluded. One hundred and twenty-five studies were collected for further review. Of these 125 studies, 96 were excluded due to lack of information on the progression of hepatitis C or lack of HCV infection control group. The remaining 29 studies[9,15-42] were included in the analysis (Figure 1A).

Study quality

The general characteristics of these studies and their

Table 1 Characteristics of these studies and their participating subjects

Reference country	Years (data collected)	Study design	Characteristics of patients	Total Patients infected with HIV-/HIV+	Duration of HCV infection HIV+/HIV- (yr)	Outcome	Covariates
USA[15]	1982-1991	Prospective cohort	Hemophilia; mean age 23 yr (2-69), 93% males, 97% whites	58/91	10-25	Liver failure	Liver function, CD4 count, excluding positive HBsAg
UK[16]	1979-1993	Retrospective cohort	Hemophilia, mean age 34 yr	109/74	15	Death, DLD	Age, type of hemophilia, CD4, 2%HBsAg+
UK[17]	1968-1995	Prospective cohort	Hemophilia, mean age 38.3 yr, 92% males, mean age of HCV infection 21 yr, follow-up time 28 yr	102/36	UK	Death	type of hemophilia, 2%HBsAg+, Pre-HAART
Italy[18]	1989-1994	Multicentre cohort	Voluntary liver biopsy patients; HIV-HCV coinfection, mean age 28 yr, 77% males, 97%IDU. HCV-infection: mean age 37 yr, 62% males, main routes of IDU and transfusion	431/116	11.3/7.6	Histological cirrhosis	Duration of HCV infection, HCV-VL, CD4, excluding alcohol, other hepatitis virus infection
Germany[19]	1989-1995	Retrospective	Voluntary liver biopsy patients; HIV-HCV coinfection, mean age 34 yr, 95% males. HCV-infection, : mean age 42 yr, 70% males	33/22	> 10	Histological fibrosis/ cirrhosis	Age, sex, CD4, 2 cases of HBsAg+
Italy[20]	1992-	Prospective Multicentre	Hemophilia, 98% males	243/141	> 20	HCC, cirrhosis, death	Age, type of hemophilia,
France[21]	UK	Retrospective	IDU, mean age 33 yr, 75% males, 35%Etoh	150/60	11.8/12.3	Histological cirrhosis	duration of HCV, 7.5%HBsAg+ EtOH
Canada[22]	1982-1998	Prospective, cohort	Hemolphilia, mean age 22.2/19.7 yr	53/81	17	Death	Type of hemophilia,
France[12]	1995-1998	Retrospective	Voluntary liver biopsy patients, 90%IDU, mean age 35.5 yr, 72% males	122/122	13	Histological fibrosis/ cirrhosis	pre-HAART era, 1 HBsAg+ Sex, Etoh, age of HCV infection, CD4, excluding HBsAg+
UK[23]	1985-1999	Prospective,	Hemophilia, mean age 17 yr	185/125	17	Death	HCV genotype, age of HCV infection, Etoh, 6 cases of HBsAg+
France[24]	1980-1995	Retrospective	IDU, mean age 31 yr, 73% males, HIV+ group: 62%IFN treatment. HIV- group: 77% IFN treatment	80/80	10.2/10.6	Cirrhosis, death	Alcohol, HCV genotype
USA[25]	1978-1999	Prospective	Hemaphilia, 96.2% whites	72/85	UK	DLD, death	HbsAg, alcohol
Greece[26]	1981-1987	Prospective	Hemophilia, 90% males, most whites, mean age of HIV+ 21 yr, mean age of HIV-18yr	624/1194	UK	DLD	Age, alcohol consume, CD4 count, duration of HCV infection, 7% HBsAg+
UK[27]	1994-2002	Retrospective	Voluntary liver biopsy patients, mean age 38.8 yr, 73% males, 77%IDU	153/55	23/21	Histological fibrosis/ cirrhosis	Sex, age of HCV infection, Etoh, HAART, excluding HBV
Spain[28]	1998-2001	Cross-section	Voluntary liver biopsy patients, mean age 40 yr, 75% males, genotype 1, IDU 88% of positive HIV, 88%HIV with ARV treatment	75/75	UK	Histological fibrosis/ cirrhosis	Sex, age of HCV infection, HAART, HCV-VL, excluding alcohol, HBsAg
France[29]	2000.4-2000.12	Prospective	Most of IDU, mean age 38 yr, most of genotype 3 or 4, 67% males, 11% with alcohol consume	33/33	15/14	Histological fibrosis/ cirrhosis	HCV genotype, HCV-VL, CD4, HIV-VL, excluding alcohol, HBsAg
China[30]	2001-2003	Retrospective	CHC of inpatients, all blood transfusion. HIV+ group, : mean age 38 yr, 50% males. HIV-group 39yr, 39% males, pre-HAART era	33/140	< 15	Clinical cirrhosis	CD4 count, excluding alcohol, HBsAg
USA[31]	1997-2004	UK	CHC, main genotype 1. IDU 76%. HIV+group: mean age 47 yr, 92% males, 15% alcohol consume. HIV-group: mean age 49 yr, 87% males, 31% alcohol consume	372/92	24/22	Histological fibrosis/ cirrhosis	HCV genotype, HCVVL, BMI, HIV-VL, CD4 count

USA[32]	UK	Prospective	Voluntary liver biopsy patients, main genotype 1, all IDU. HIV+ group: mean age 47 yr, 77% males. HIV-group: mean age 47 yr, 60% males, 83% with HAART	57/40	UK	Histological fibrosis/ cirrhosis	HCV-VL, HCV genotype, CD4, HIV-VL
USA[33]	1991-2000	Retrospective	CHC with inpatients, mean age 45 yr, 97% males, 58% without HAART	26641/4761	UK	HC, HCC	HAART
Canada[34]	1982-2003	Cohort	Hemophilia, 98% pre-HAART era	712/444	UK	Death	Type of hemophilia
Spain[35]	1997-2002	Multicentre Retrospective	Patients with decompensated HCV-related cirrhosis, HIV-infected: mean age 38 yr, 86% males, 86% IDU, HCV genotype1 63%, HbsAg 24%. HIV-uninfected:mean age 66 yr, 58% males, HCV genotype1, 83% other sources of HCV infection. 91% HIV-infected patients with HAART	1037/180	26/15	Death	Age, HCV genotype, HCV-VL, excluding HBsAg
Spain[36]	1990-2002	Prospective	IDU, 77% males, follow-up time 8.6 yr	1418/1465	9.6/13.5	Death	Age, sex, HAART, HAART, 4.6% HbsAg+
UK[37]	1961-2005	Multicentre	Hemophilia, mean age 43 yr, 94% males, HCV genotype1 53%	497/190	27	DLD	Age, alcohol consume, HCV genotype,HAART, 2.8%HBsAg+
Zambia[38]	2000-2004	Retrospective	HIV-infected: mean age 38 yr, 76% males, 50% IDU. HIV-unifected: mean age 48 yr, 51% males, 31% blood transfusion. 91% HIV patients with HAART, time of HAART 3.6 yr	247/162	19.8/15	Histological fibrosis/ cirrhosis	Age, sex, alcohol consume, HCV-VL, excluding HbsAg+
USA[39]	1999-2002	Retrospective	HIV+ :mean age 45 yr, 80% males, 72% IDU. HIV-: mean age 48 yr, 79% males, 58% IDU. 79% genotype 1. 95% HIV patients with HAART. time of HAART 3.6 yr	382/274	25/23	Histological fibrosis/ cirrhosis	Age, alcohol consume, HCV genotype, HCV-VL, CD4, HIV-VL, excluding HBsAg
Dutch[40]	1985-2006	Prospective	IDU, mean age 30 yr, 64% males, follow-up time 9 yr (5-14)	565/256	8/10	Death	HAART, CD4
Zambia[41]	2003-2004	Retrospective	HIV+: mean age 40 yr, 79% males, 79% IDU. HIV-: mean age 46 yr, 45% males, 25% IDU	65/53	18.7/20.6	Histological fibrosis/ cirrhosis	Liver function, HCV-VL, HCV genotype, CD4, excluding HbsAg, patients with DLD
France[42]	2004-2006	Retrospective	HIV+ mean age 43 yr, 67% males, 83% IDU HIV-: mean age 52 yr, 38% males, 45% blood transfusion; 88% HIV patients with HAART	656/287	23.5/22.1	Fibroscan of fibrosis/ cirrhosis	Age, sex, BMI, HCV-subtype, HCV-VL, HIV-VL, CD4 count excluding HbsAg+, alcohol

DLD: Decompensated liver disease; HCC: Hepatocellular carcinoma; IDU: Injection drug user; HAART: Highly active antiretroviral therapies; Etoh: Ethyl alcohol.

participating subjects are shown in Table 1. The number of patients participating in the studies ranged 55-2883. Their mean age was 21-50 years. Most patients were men. Of the 29 cohort studies, 13 were retrospective in design, 11 were prospective in design, and 1 was a cross-study in design, and 4 did not show the type of design. We evaluated data of 16750 HCV-positive patients. Of them, 6242 were positive for HIV and 10508 were negative for HIV. Fourteen studies assessed the histological cirrhosis[9,18,19,21,27-32,38,39,41,42], 7 studies assessed the death[17,22,23,34-36,40], 3 studies assessed the decompensated liver disease[15,26,37], 2 studies assessed the outcome of decompensated liver disease and death[16-25], 1 study assessed the outcome of histological cirrhosis, hepatocellular carcinoma and death[20], 1 study assessed the outcome of histological cirrhosis and death[24], and 1 study assessed the outcome of hepatocellular carcinoma and death[33], respectively. Most studies did not provide a racial distribution. There was a similar variability in HCV viral load.

Studies in immunocompetent persons showed that the progression of chronic HCV infection is affected by many external and host factors, including duration of HCV infection, alcohol consumption, coinfection with other hepatitis viruses, *etc*. Other studies assessed the duration of hepatitis C except for 6 studies[25,26,28,32-34]. In

these studies, HCV infection was typically assumed due to the exposure to clotting factor, blood transfusion, or initiation of injecting drugs. The mean duration of HCV infection ranged 10-28 years. In our analysis, 3 studies excluded patients who consumed alcohol excessively, 15 studies attempted to assess the significance of alcohol use by quantifying grams of alcohol consume per day, the other studies did not show alcohol consume of patients. Because of the various methods to describe alcohol consumption in these studies, this important factor could not be incorporated into further analyses. Twelve studies excluded patients with detectable hepatitis B surface antigen from their cohort, 4 studies did not describe the state of hepatitis B surface antigen in their cohort, the other 12 studies reported the number of patients with positive HBV surface antigen (Table 1).

Meta-analysis

Inclusion of all end points in studies: The combined unadjusted OR for the 29 studies was 3.40 (95% CI = 2.45 and 4.73) by the random effect model (Figure 1A and B). The test for heterogeneity was significant ($P < 0.001$). Since some factors led to the significant heterogeneity, including different outcomes of our analysis, duration of HCV infection, we also performed subgroup analyses determined a priori.

Analysis of the end points of histological cirrhosis and liver cancer: Seventeen studies assessed liver fibrosis or cirrhosis in patients with HIV-HCV coinfection and HCV infection. The outcome of cirrhosis in in studies was confirmed by histological diagnosis. All studies addressed the effect of duration of HCV infection on progression to severe liver disease. Since the test for heterogeneity had no statistical significance ($P = 0.15$), the fixed effect model was used for subsequent analyses. Thirteen studies examining the end point of histological cirrhosis had a pooled OR of 1.47 (95% CI = 1.27 and 1.70). Cirrhosis was stratified by duration of HCV infection in years. The combined OR for duration of HCV infection within 15 years was 1.80 (95% CI = 1.41 and 2.30) in 6 studies, whereas that for duration of HCV infection exceeding15 years was 1.33 (95% CI = 1.11 and 1.59) in 7 studies (Figure 1B). Five studies examined the end point of decompensated liver disease (DLD). The test for heterogeneity was significant ($P = 0.008$). The random effect model was used for subsequent analyses. The pooled OR for DLD was 5.45 (95% CI = 2.54 and 11.71, Figure 1C). Only 2 studies assessed the end point of hepatocellular carcinoma. The pooled OR for liver cancer was 0.76 (95% CI = 0.50 and 1.14, Figure 1D).

Analysis of end point of death: Eight studies assessed the end point of death in the two groups before HAART. The combined OR for the 8 studies was 3.60 (95% CI = 3.12 and 4.15) using the fixed effect model. Death was stratified by duration of HCV infection in years. The combined OR for duration of HCV infection within 15 years was 2.95 (95% CI = 2.52 and 3.46), whereas that for duration of HCV infection exceeding 15 years was 9.01(95% CI = 6.35 and12.77, Figure 1F).

DISCUSSION

Our meta-analysis quantitatively assessed the influence of HIV infection on the course of HCV infection. The overall OR for histological cirrhosis or decompensated liver disease or liver cancer or death was 3.40 (95% CI = 2.45 and 4.73) by the random effect model. However, these studies had a significant heterogeneity. Some factors could explain the heterogeneity, including different outcomes in these studies, methodological differences in study design, different number of patients in cohort, bias in selection of patients for biopsies, effect of duration of HCV infection on progression to severe liver disease, and publication bias.

We also performed subgroup analyses to determine the priority according to the different outcomes and duration of HCV infection. Striking differences were found in studies examining different end points of histological cirrhosis, decompensated liver disease, hepatocellular carcinoma or death. The combined adjusted OR for histological cirrhosis, decompensated liver disease, hepatocellular carcinoma and death was 1.47 (95% CI = 1.27 and 1.70), 5.45 (95% CI = 2.54 and 11.71), 0.76 (95% CI = 0.50 and 1.14), and 3.60 (95% CI = 3.12 and 4.15), respectively. There was a smaller difference between HIV-HCV coinfected and HCV-infected patients with regard to the development of cirrhosis or liver cancer, but there was a substantial increased risk of developing decompensated liver disease or death.

In the pre-HAART era, many patients coinfected with HIV and HCV died of opportunistic infection, lymphoma or wasting syndrome due to severe immunodeficiency, which is the main risk factor for death of HIV-HCV coinfection patients. The declined HIV-related mortality after widespread use of HAART parallels the emergence of HCV-related liver disease as an important cause of mortality in coinfected patients. Studies indicate that HAART has a protective effect on fibrosis progression in patients with HIV-HCV coinfection. On the other hand, HAART may enhance liver damage in some HIV-HCV coinfected individuals through drug-related hepatotoxicity. Only 6 studies[28,32,35,38,39,42] in our analysis introduced the state of HAART in patients with HIV-HCV co-infection. The proportion of patients who were receiving HAART in cohort ranged 83%-95%. However, we could not acquire the primary data comparing progression of HCV infection in pre-HAART and HAART era, and the other studies were performed before the widespread use of HAART. We, therefore, did not examine the impact of HAART on the progression of HCV infection. This is an important limitation in our study and its impact on the progression of HCV-induced liver disease needs to be explored.

Other factors may have influenced the level of liver damage in HIV-HCV coinfected patients. Important fields for further study include the effects of HAART on HCV-related liver disease progression, duration of

HCV infection, and alcohol consumption.

The results of our study suggest that HIV infection can significantly change the natural history of HCV infection, especially in the development of death or decompensated liver disease. Because most cohorts in our analysis were composed of patients with hemophilia or injection drugs, and most patients studied were males, caution should be taken in generalizing the results of this meta-analysis of women and racial or ethnic populations not represented in these studies. All these factors may potentially impact the natural history of HCV infection.

COMMENTS

Background

Hepatitis C virus (HCV) infection is a major public health problem. Coinfecion with human immunodeficiency virus (HIV) and HCV frequently shares blood, sexual, mother-to-child routes of transmission. It is estimated that 4-5 million patients were coinfected with HIV and HCV in the world. HCV is the leading non-AIDS cause of death. A large number of cohort studies have examined the different impacts of HIV on HCV infection in terms of clinically unambiguous end points of decompensated liver disease and biopsy-proven cirrhosis.

Research frontiers

Interaction of HIV and HCV is a hot spot. HIV infection can change the natural history of chronic hepatitis C with an unusually rapid progression to cirrhosis. HIV-related immunodeficiency may be a determinant factor for higher hepatitis C viremia levels and more severe liver damage.

Innovations and breakthroughs

This study analyzed the impact of human immunodeficiency virus infection on the course of HCV infection, and compared the clinically unambiguous end points of decompensated liver disease, cirrhosis, hepatocellular carcinoma and death.

Applications

Meta-analysis suggests that HIV accelerates HCV disease progression, death, histological fibrosis/ cirrhosis and decompensated liver disease. However, the rate of hepatocellular carcinoma is similar in patients infected with HCV who are positive or negative for HIV. This has important implications for the timely diagnosis and treatment of patients coinfected with HCV and HIV.

Peer review

This manuscript is reasonably well written and contains interesting information.

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CASE REPORT

Severe autoimmune hepatitis triggered by varicella zoster infection

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Abstract

Autoimmune hepatitis (AIH) is a chronic disease of unknown etiology that is characterized by the presence of circulatory autoantibodies and inflammatory histological changes in the liver. Although the pathogenesis of AIH is not known, it is thought that, in a genetically predisposed individual, environmental factors such as viruses can trigger the autoimmune process. Herpes simplex virus, Epstein-Barr virus, measles virus, and hepatitis viruses are thought to play a role in the etiology of AIH. Proteins belonging to these viruses may be similar to the amino acid chains of different autoantigens in the liver, this causes immune cross reactions and liver tissue damage. We report a case of severe AIH following varicella zoster infection in a 23-year-old man, and speculate that, based on the molecular mimicry hypothesis, the liver damage was caused by an immune cross reaction to the viral proteins. Varicella-zoster-induced AIH has not been reported previously.

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Key words: Autoimmune liver disease; Varicella zoster virus; Infection

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INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic disease of unknown etiology that is characterized by the presence of circulatory autoantibodies and inflammatory changes in liver histology[1]. Several triggers for AIH, particularly drugs and viral hepatitis, have been described, which may induce the development of autoimmunity in predisposed individuals[1,2]. We report a case of severe AIH preceded by varicella zoster virus (VZV) infection, which we believe, triggered the AIH. The possible pathogenic mechanism is based on the molecular mimicry hypothesis, in which viral proteins that are similar to the amino acid chains of autoantigens in the liver induce immune cross reactions that cause liver damage[3]. As far as we are aware, VZV-induced AIH has not been reported previously.

CASE REPORT

A 23-year-old man was referred to our hepatology service on September 1, 2007 with jaundice, anorexia, weight loss and malaise of 2 mo duration. The episode of jaundice was preceded by VZV infection (chicken pox), which he contracted from close contact with infected family members. Despite recovery from the skin eruption, he had persistent anorexia and intermittent right upper quadrant pain. One month later he developed increasing jaundice. He had no prior history of alcohol ingestion or substance abuse and no family history of liver disease. His past medical history was otherwise unremarkable. His initial presentation to our hospital was 2 mo following the onset of jaundice. His physical examination revealed jaundice and hepatomegaly 2 cm below the costal margin. His initial laboratory findings were as follows: hemoglobin 149 g/L, white blood cell count 9.9 × 10^9/L, platelets 512 × 10^9/L, erythrocyte sedimentation rate 39 mm/h, alanine aminotransferase (ALT) 1066 U/L, aspartate aminotransferase (AST) 755 U/L, alkaline phosphatase (ALP) 185 U/L, total bilirubin 425 μmol/L, direct bilirubin 318 μmol/L, and albumin 32 g/L. His globulins were elevated at 46 g/L and his IgG was also elevated at 20.5 g/L. The coagulation profile was normal. Anti-

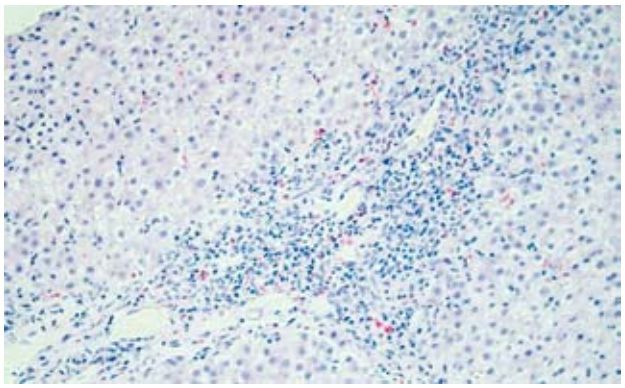

Figure 1 Portal tract expansion secondary to infiltration by inflammatory cells (interface hepatitis is seen). (HE stain × 10).

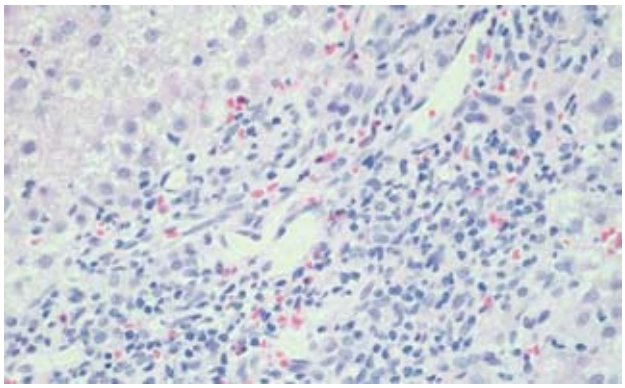

Figure 2 Inflammatory cells comprising mainly lymphocytes with some plasma cells. (HE stain × 20).

nuclear, anti-smooth muscle, anti-mitochondrial and anti-liver/kidney microsomes (ALKM-1) autoantibodies were negative. Perinuclear antineutrophil cytoplasmic antibodies were positive. Hepatitis B surface antigen, anti-hepatitis B core antigen IgM, anti-hepatitis C virus antibodies, anti-cytomegalovirus (CMV) IgM, anti-Epstein-Barr virus (EBV) IgM, and anti-hepatitis A virus (HAV) IgM were all negative. Anti-VZV IgG antibodies were positive. Ceruloplasmin and iron indices were normal.

The patient refused a liver biopsy, therefore, we started him on a tapering course of prednisone for presumed AIH, at a starting dose of 60 mg. He responded to that dramatically, as he became totally asymptomatic and started gaining weight. Within 2 mo of treatment, his liver enzymes normalized. On tapering his steroids below 10 mg, his liver enzymes increased again. His prednisone dose was increased to 30 mg and 50 mg azathioprine was added, however, because of vomiting and epigastric pain, the latter was changed to mycophenolate mofetil 500 mg twice daily. Follow-up showed improvement in his liver enzymes. In August, 2008, he stopped all his medications as he was feeling well and his liver enzymes were normal. A follow-up visit in September, 2008 revealed an increase in his liver enzymes, with ALT of 501 U/L and AST of 230 U/L, and bilirubin and ALP remained normal. A liver biopsy was performed and revealed features of AIH, with interface hepatitis, predominant lymphocytic infiltration, and the presence of plasma cells. Additionally, bridging fibrosis was also noted (Figures 1 and 2).

Treatment was reintroduced with improvement in the biochemical markers (Figure 3).

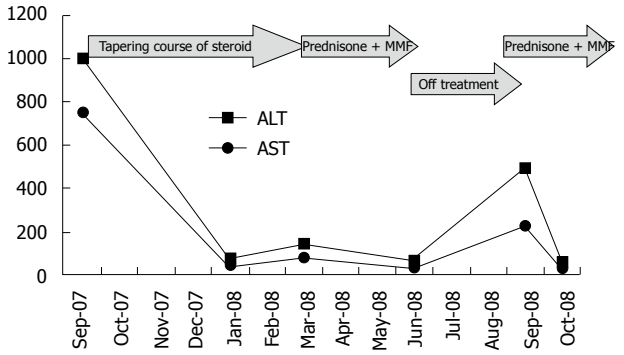

Figure 3 ALT and AST pattern during the course of the disease.

DISCUSSION

VZV is a double-stranded, linear DNA virus. Primary infection with VZV causes chicken pox in susceptible hosts[4]. Varicella hepatitis in immunocompetent hosts is usually self-limiting and asymptomatic, with subclinical elevation in serum transaminase levels[5]. However, severe varicella hepatitis leading to fulminant liver failure has been reported[6]. There is an increased risk of VZV infections in patients with underlying autoimmune disease. However, VZV-triggered autoimmune diseases have not been reported before. In the present case, anorexia and weight loss persisted following resolution of the varicella-induced skin eruption. One month later, the patient developed jaundice that persisted for 2 mo before the patient presented to our hospital. The patient had a post-treatment score of 17 according to the revised International Autoimmune Hepatitis Group system. The sequence of events in this patient indicated strongly that the AIH was triggered by VZV infection.

The pathogenic mechanism of AIH is unknown. Genetic predisposition is considered to play a major role, however, it is unlikely to be the single causative factor and other triggering factors may exist. There has been evidence implicating CMV, EBV, measles virus and hepatitis viruses as triggers of the disease[1,7-11]. It is thought that viral proteins belonging to these viruses may be similar to amino acid chains of different autoantigens in the liver, and this resemblance leads to immune cross reactions that target liver tissue. Asialoglycoprotein receptor, which is found in high density in the periportal hepatocytes, is an autoantigen that is thought to play a role in the immunological reactions in AIH[12]. A defect in suppressor-inducer T lymphocytes, specifically controlling immune responses to the asialoglycoprotein receptor, has been detected in patients following viral infection[13,14].

The study of Vento *et al*[13] supports the concept that the pathogenesis of AIH in genetically susceptible individuals is caused by an environmental trigger that

leads to an aberrant autoimmune response. They followed 53 relatives of 13 AIH patients for a total of 4 years. Three cases developed subclinical hepatitis A and two of them subsequently developed AIH after 5 mo. Prior to developing HAV, a defect in suppressor-inducer T lymphocytes specifically controlling immune responses to the asialoglycoprotein receptor was detected[13]. Following this study, multiple reports linking AIH to viral infections were published[7-11]. In addition to the molecular mimicry theory, other pathogenic mechanisms that are thought to play a role in virus-induced AIH include modification or release of sequestered cellular proteins by viruses, activation of resting T cells by inducing the release of a variety of cytokines, and polyclonal activation of lymphocytes[15].

In conclusion, VZV infection can trigger AIH that may progress to advance liver fibrosis and eventually cirrhosis in genetically predisposed individuals. Therefore, AIH should be considered in any patient with persistently altered liver enzymes following a viral infection. Similarly, patients with AIH should be screened for viral hepatitis.

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CASE REPORT

Abscess in the inguinal hernial sac after peritonitis surgery: A case report

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Abstract

In this paper, we report an extremely rare case of an abscess that developed in the inguinal hernial sac after surgery for peritonitis. A 60-year-old man underwent laparoscopic low anterior resection for rectal cancer. One day after this operation, peritoneal drainage and ileostomy were performed for rectal anastomotic leakage. Five days after the second operation, computed tomography revealed an abscess in the left inguinal hernial sac. Subsequently, hernioplasty and resection of the inflamed sac were performed.

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Key words: Inguinal hernia; Hernial sac abscess; Peritonitis; Anastomotic leakage, Rectal cancer

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Ikeda S, Takeda H, Yoshimitsu M, Hinoi T, Yoshida M, Sumitani D, Takakura Y, Kawaguchi Y, Shimomura M, Tokunaga M, Kawahori K, Ohdan H, Okajima M. Abscess in the inguinal hernial sac after peritonitis surgery: A case report. *World J Gastroenterol* 2009; 15(8): 1007-1009 Available from: URL: http://www.wjgnet.com/1007-9327/15/1007.asp DOI: http://dx.doi.org/10.3748/wjg.15.1007

INTRODUCTION

Collections of infected ascites can remain in recesses of the peritoneal cavity after peritonitis. This may lead to inflammation and the formation of an abscess in the hernial sac. The condition of hernial abscess secondary to peritonitis was first reported by Cronin *et al*[1], and since then, a few other cases have been reported. Although neither peritonitis nor hernia is a rare disease, surgeons do not sufficiently consider the possibility of hernial sac abscess. Here, we present a case in which an inguinal hernial abscess developed following surgery for peritonitis caused by rectal anastomotic leakage.

CASE REPORT

A 60-year-old man underwent laparoscopic low anterior resection for rectal cancer. The intraoperative laparoscopic examination revealed that the patient had a left inguinal hernia, and that a tip of the omentum had extended into the left hernial sac through the deep inguinal ring (Figure 1). The omentum was removed from the hernial sac by using ultrasonic coagulating shears. However, the inguinal hernia was not repaired during the operation. Anastomotic leakage and peritonitis were observed 1 d after the first operation. The patient underwent emergency peritoneal drainage and ileostomy. On day 6 after the first operation (i.e. day 5 after the second operation), the patient noticed a tender mass in the left groin lesion and developed fever. The results of a blood examination revealed elevated C-reactive protein and a high white blood cell count. A computed tomography (CT) scan showed a mass with a diameter of 10 cm, accompanied by inflammation, in the left groin lesion (Figure 2). The mass was suspected to contain liquid and fat, but not bowel tissue. It was

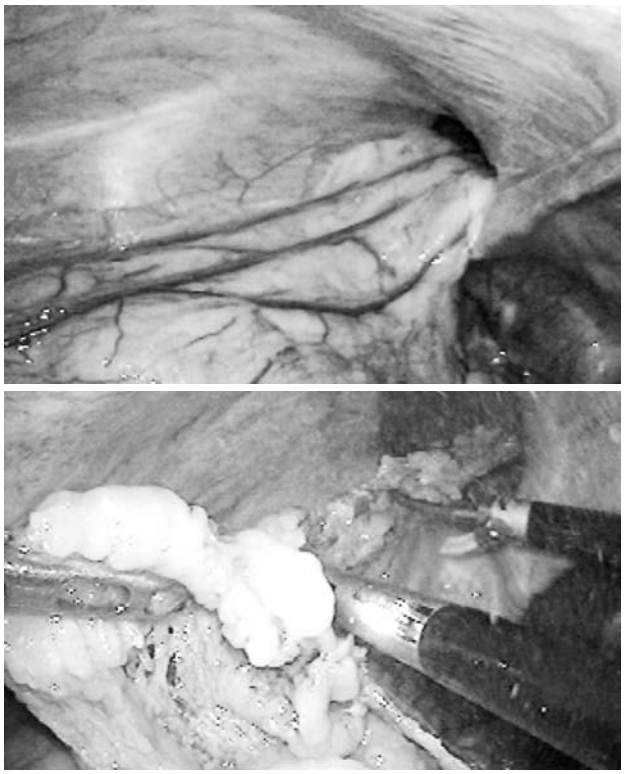

Figure 1 Intraoperative laparoscopic examination performed during surgery for rectal cancer revealed protrusion of a part of the omentum into the left inguinal hernia.

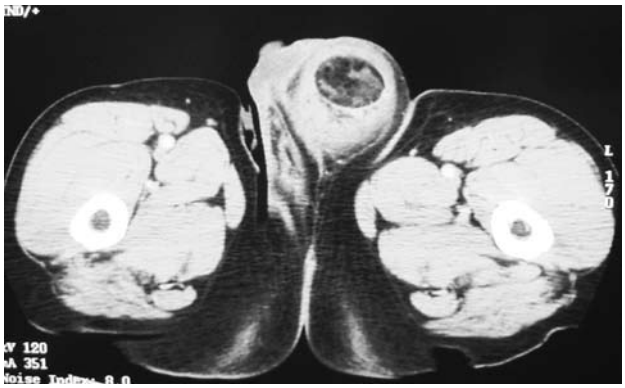

Figure 2 CT showed an inflamed mass in the left groin lesion.

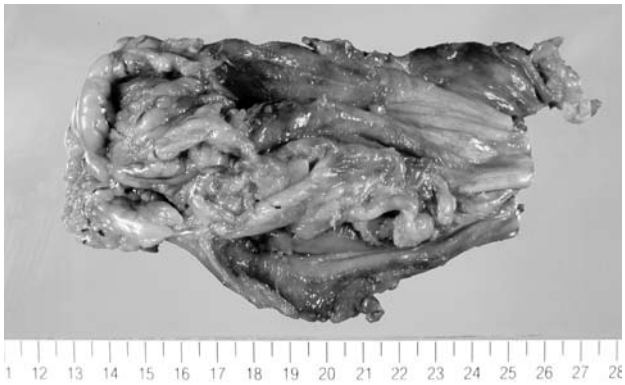

Figure 3 Necrotic fat tissue was observed in the resected hernial sac.

diagnosed as an abscess in the inguinal hernial sac, and resection of the sac and iliopubic tract repair were preformed. The wall of the hernial sac had thickened and extended into the left scrotal sac through the posterior region of the inguinal ligament. Infected ascites and inflamed necrotic fat tissue, suspected to be remnants of the omentum, were observed in the hernial sac (Figure 3).

DISCUSSION

Inguinal hernia and peritonitis with various underlying etiologies are not rare conditions. However, hernial sac abscess associated with peritonitis is very rare. In this paper, we report a case in which an abscess developed in the left inguinal hernial sac following peritonitis caused by rectal anastomotic leakage.

Several cases of abscess in the hernial sac caused by acute appendicitis or by strangulation and perforation of the intestines in the hernial sac have been reported[2,3]. The protrusion of a vermiform appendix into the inguinal hernial sac, termed an Amyand hernia, and into the femoral hernial sac is termed a De Garengeot hernia[4,5]. When acute appendicitis occurs in the hernial sac, it may lead to the development of a hernial sac abscess[3]. However, our review of the literature revealed that, after cases of abscess in the hernial sac secondary to peritonitis were reported by Cronin and Ellis in 1959 and Chary in 1977[1,6], few other such cases have been reported. Sakata *et al*[7] have reviewed eight reports that describe cases of hernial abscesses secondary to peritonitis, and have classified these conditions into two groups: immediate and delayed hernial abscesses. Immediate hernial abscesses occur during the acute phase of peritonitis, and surgery for the abscesses and peritonitis are often performed simultaneously. On the other hand, delayed abscesses develop after surgery for peritonitis. According to this system of classification, the case reported here is a delayed hernial abscess because it was first noted 5 d after surgery for peritonitis. In several previously reported cases, the sac abscess was noticed without a certain diagnosis of peritonitis before operation[8-11]. This may have been caused by the lack of efficient diagnostic modalities, such as CT, in the past. However, presently, definitive preoperative diagnosis of peritonitis can be assured, as a result of techniques such as CT and abdominal ultrasonography that are easy to perform and provide images of not only the hernial sac but also the abdominal cavity.

In our patient, infected ascites accumulated in the inguinal hernial sac because of rectal anastomotic leakage may have remained after surgery for peritonitis. Further, it is possible that the proliferation of bacteria in the sac may have led to the development of the abscess; however, this possibility was not examined because we did not perform culture of the contents of the hernia abscess. In addition, the omentum, which was partially and insufficiently removed during the operation for rectal cancer, may have played an important role in triggering the inflammation in the sac. Examination

of the resected hernial sac revealed that the remnant omentum developed necrosis, possibly because the blood supply to this tissue was intercepted during the operation. The presence of the necrotic omentum in the sac may have rendered the environment conducive to bacterial proliferation in the sac, thus resulting in inflammation and the development of an abscess.

The most important issue is that of whether surgeons should perform an additional operation for hernia detected during surgery. In the present case, we had two opportunities to perform an operation for the inguinal hernia; the first during the operation for rectal cancer and the second during the operation for peritonitis caused by anastomotic leakage. However, we did not perform the operation for hernia on either of these occasions. Hernioplasty with a prosthetic mesh should not be performed during an operation for rectal cancer because the surgical site is not sterile, and this leads to bacterial contamination of the prosthetic mesh. Further, it is obvious that the use of a prosthetic mesh in an operation for peritonitis should be avoided. A more appropriate alternative would be to perform extra-peritoneal laparoscopic hernia repair or tension-free open hernia repair during surgery for rectal cancer and to perform hernioplasty separately. Although we can not definitively state the best method for managing hernia detected during peritonitis surgery, we believe that it is important to adequately wash the hernial sac, as well as the peritoneal cavity with saline, and to administer appropriate antibiotics after the operation[7]. Furthermore, we believe that careful observation of the groin lesion after peritonitis surgery is important in patients with a hernial sac.

In conclusion, we have presented a rare case in which an inguinal hernial sac abscess developed after peritonitis. It is necessary for surgeons to carefully consider the possibility of detecting a hernial sac abscess during surgery for peritonitis and during postoperative follow-up.

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CASE REPORT

Pancreatic tuberculosis masquerading as pancreatic serous cystadenoma

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Abstract

Solitary pancreatic involvement of tuberculosis is rare, especially in an immunocompetent individual, and it may be misdiagnosed as pancreatic cystic neoplasms. Pancreatic cystic neoplasms are being identified in increasing numbers, probably because of the frequent use of radiology and advances in endoscopic techniques. However, they are composed of a variety of neoplasms with a wide range of malignant potential, and it is often difficult to differentiate pancreatic tuberculosis mimicking cystic neoplasms from benign or malignant pancreatic cystic neoplasms. Non-surgical diagnosis of pancreatic tuberculosis is inconclusive and continues to be a challenge in many cases. If so, then laparotomy should be employed to establish the diagnosis. Therefore, pancreatic tuberculosis should be kept in mind during the differential diagnosis of solitary cystic masses in the pancreas. We report a patient who had solitary pancreatic tuberculosis masquerading as pancreatic serous cystadenoma.

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Key words: Tuberculosis; Pancreas; Cystadenoma; Serous neoplasm

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INTRODUCTION

Pancreatic tuberculosis is uncommon and isolated involvement is rare. Pancreatic tuberculosis is a well-known masquerader, and the diverse clinical and radiological picture of this chronic infection may be revealed as a variable pancreatic lesion, including pancreatic cystic neoplasm, such as serous cystadenoma. In view of the non-specific presentation and imaging appearance of the disease, a high index of suspicion is required to obtain a preoperative diagnosis. However, the diagnosis is usually established at laparotomy. Herein, we describe one case of solitary pancreatic tuberculosis that presented as a lobulated multicystic neoplasm that resembled serous cystadenoma.

CASE REPORT

A 51-year-old woman was referred from another hospital for further management of a pancreatic head tumor detected on an abdominal ultrasound scan. On admission, the patient appeared to be in excellent condition. She presented with mild epigastric discomfort which had persisted for 2 wk. She had no tenderness over the epigastric area and no definite mass was palpable. Other physical examination findings were unremarkable. According to the patients' history, she had no coughing, fever, jaundice, diarrhea, hematemesis or melena. There was no prior history of pancreatitis, liver disease, alcohol use, tuberculosis or malignancy in the patient or her family. Initial laboratory values revealed: white blood cell count, 6.9 $\times$ 10^9/L; hemoglobin, 11.4 g/dL; aspartate aminotransferase, 19 IU/L (reference range, 10-44 IU/L); alanine aminotransferase, 6 IU/L (12-79 IU/L); total bilirubin, 0.9 mg/dL (0.2-1.3 mg/dL); albumin, 4.0 g/dL (3.3-5.1 g/dL); alkaline phosphatase, 8 IU/L (42-136 IU/L); amylase, 69 U/dL (28-100 U/dL); and lipase,

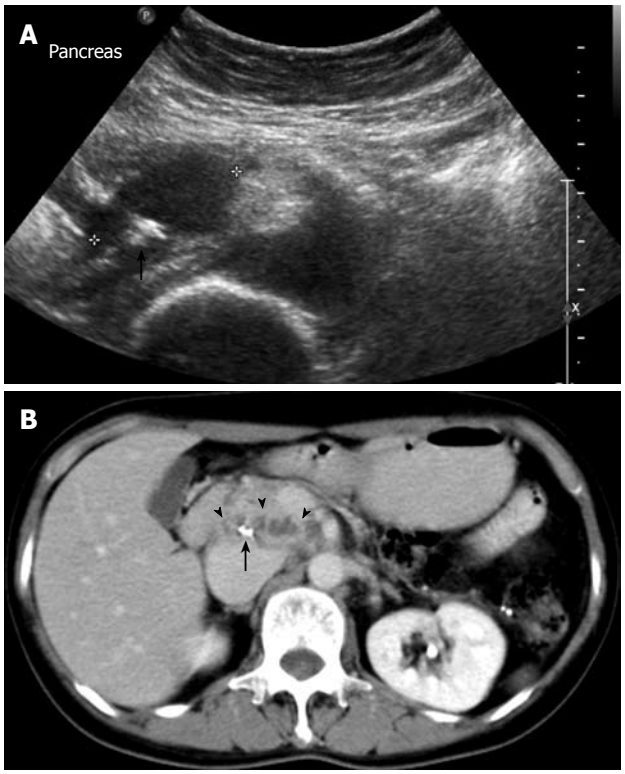

Figure 1 Abdominal US and CT at admission. A: Abdominal US revealed an irregularly contoured, hypoechoic, cystic lesion in the head of the pancreas, with calcification at the center of the mass (arrow); B: abdominal CT demonstrated an inhomogeneous lobulated multicystic mass of 4.5 cm × 2.0 cm in the head and uncinate process of the pancreas, with central calcification (arrow).

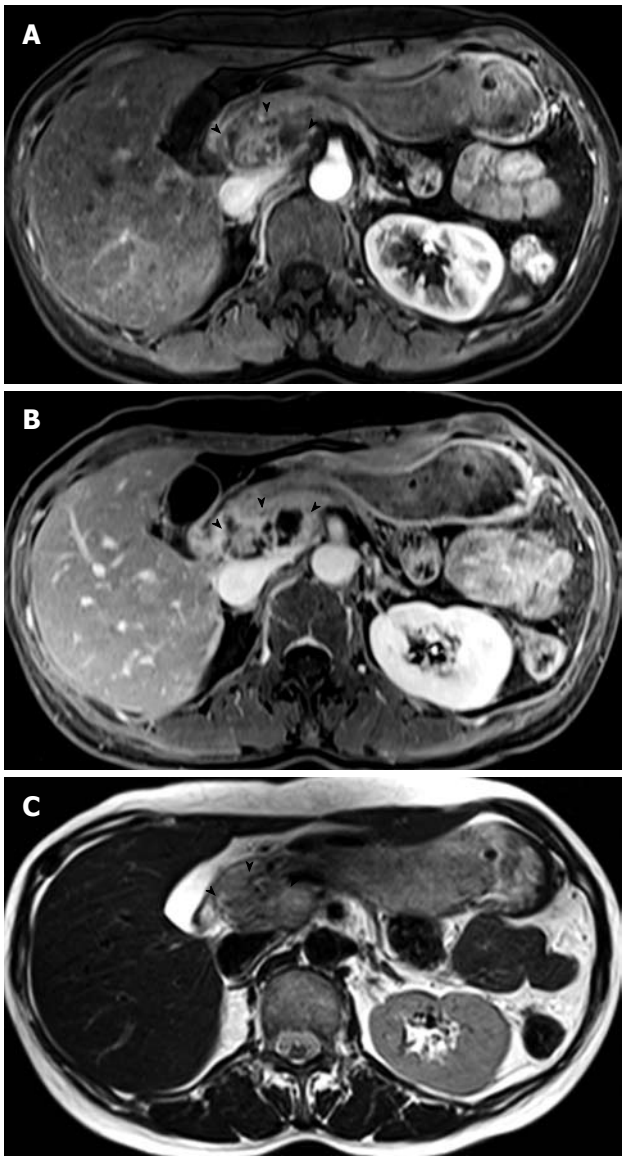

Figure 2 MRI of the pancreatic mass. A and B: Gadolinium-enhanced T-1 weighted image of the sharply delineated multiloculated mass in the pancreas head, with peripheral and central areas of enhancement; C: T-2 weighted image of the heterogeneous mass with increased and decreased signal intensities.

80.3 U/dL (10-150 U/dL). Serological tests for antibodies to hepatitis B virus surface antigen, hepatitis C virus, and human immunodeficiency virus yielded negative results. Other blood tests were normal, except for an elevated erythrocyte sedimentation rate (99 mm/h). Levels of carbohydrate antigen 19-9 and carcinoembryonic antigen were within normal limits. A chest radiograph exhibited no abnormal findings. Abdominal ultrasound (US) examination revealed an irregularly contoured, hypoechoic, cystic lesion in the head of the pancreas, with calcification at the center of the mass and no dilation of the bile duct system or the pancreatic duct (Figure 1A). Contrast-enhanced computerized tomography (CT) of the abdomen showed an inhomogeneous lobulated multicystic mass of 4.5 cm × 2.0 cm in the head and uncinate process of the pancreas, with central calcification (Figure 1B). Subsequent magnetic resonance imaging (MRI) revealed a sharply delineated multiloculated mass in the pancreas head with peripheral and central areas of enhancement on a gadolinium-enhanced T-1 weighted image (Figure 2A and B). On the T-2 weighted image, a heterogeneous mass with areas of increased and decreased signal intensities was noted (Figure 2C). Endoscopic US also demonstrated a lobulated multicystic lesion of heterogeneous echotexture (Figure 3A). Endoscopic retrograde cholangiopancreatography (ERCP) showed a normal appearance of the ampulla and no mucus secretion from its orifice (Figure 3B). There was no communication between the pancreatic duct and the cystic mass (Figure 3C). After completing these investigations, the principal provisional diagnosis of the lesion was a cystic neoplasm of the pancreas, such as serous cystadenoma.

We planned close observation and follow-up by regular surveillance imaging because malignant transformation of serous cystadenoma is exceedingly rare. However, the patient and her family preferred removal of the lesion because they were concerned about its malignant potential. She underwent a pylorus-preserving Whipple's operation (pancreaticoduodenectomy). The operation revealed a firm mass in the head of the pancreas, approximately 6.5 cm × 4.0 cm × 3.8 cm in size, and lymphadenopathy. Histological examination of the resected specimen showed caseating granulomas and the presence of Langhans' giant cells, which was suggestive of tuberculosis in the pancreatic head and lymph nodes, and polymerase chain reaction for tuberculosis was

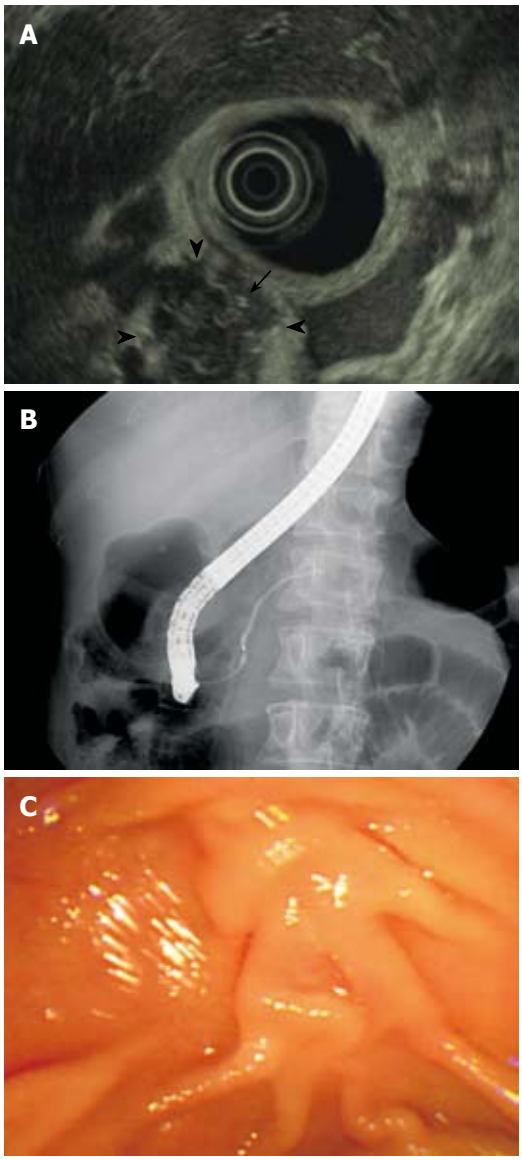

Figure 3 EUS and ERCP findings. A: Lobulated multicystic lesion of heterogeneous echotexture and focal calcification (arrow); B: endoscopic view of the normal appearance of the ampulla with no mucus secretion from its orifice; C: fluoroscopic view of no communication between the pancreatic duct and the cystic mass.

positive (Figure 4). Following the histological diagnosis of pancreatic tuberculosis, the patient was successfully treated with antituberculous therapy. The patient is doing well and has experienced no further abdominal discomfort.

DISCUSSION

Tuberculosis is still a common illness worldwide. Dissemination to the gastrointestinal tract, liver, spleen and mesenteric lymph nodes is common in abdominal and military tuberculosis in developing countries. Although extra-pulmonary tuberculosis is an emerging clinical problem, it rarely affects the pancreas.

Pancreatic tuberculosis usually occurs as a complication of miliary tuberculosis in immunodeficient individuals. Isolated involvement of the pancreas is exceedingly

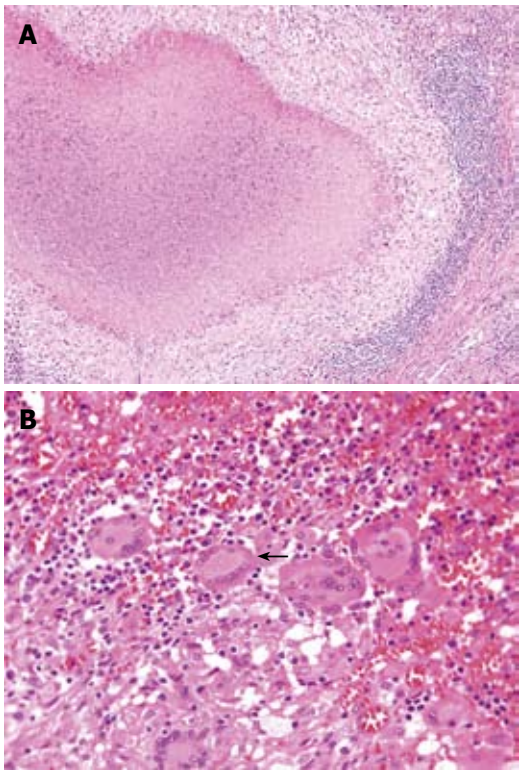

Figure 4 Histological image of resected specimen. A: Photograph showing the epithelioid granuloma with central caseous necrosis (HE, original magnification, × 100); B: multinucleated Langhans' giant cell with surrounding lymphocytes (arrow) (HE, original magnification, × 400).

rare worldwide[1,2]. It presents radiologically with variable manifestations, with a wide spectrum of symptoms, and can mimic pancreatic cystic neoplasm[3]. CT may show an enlarged pancreas with focal hypodense lesions and irregular borders, sometimes with an irregular multilobular cyst in the head region or enlarged peripancreatic lymph nodes[4]. In contrast-enhanced CT, this well-defined mass may show irregular margins with peripheral enhancement and multiloculated appearance of central enhancement. These features, however, are non-specific and may resemble those of neoplastic cystic lesions of the pancreas. The MRI features of focal tuberculosis of the pancreas show a sharply delineated mass located in the pancreatic head, which shows heterogeneous enhancement[5]. However, there are no radiological features that are pathognomonic of tuberculosis. The diagnosis usually is not suspected before laparotomy unless there is evidence of tuberculosis elsewhere, or a relevant clinical history[4,6,7].

Serous cystadenoma, also known as microcystic adenoma, is usually a benign pancreatic neoplasm that occurs most often in women and is typically diagnosed during the sixth to ninth decades of life[8]. Usually small (< 2 cm) and microcystic, they may grow to be quite large. The most common feature of serous cystadenoma is a polycystic pattern. The cystic lesion has many loculi, thin septa, external lobulation, and central scarring with stellate calcification upon CT[9]. External lobulation and a central scar, with or without a stellate pattern of calcification, are two important morphological features. The

presence of a central scar visualized with the use of CT or MRI is a highly diagnostic feature that is found in about 30% of serous cystadenomas. The finding of multiple small (< 3 mm) compartments within a cystic lesion in endoscopic ultrasound images is suggestive of serous cystadenoma[10]. A cyst with a central stellate scar is considered virtually diagnostic of a serous cystadenoma.

This radiological appearance of pancreatic cystic tumors is excellent and, owing to recent improvements in pancreatic imaging, increasing numbers of cystic lesions have been identified in asymptomatic patients. However, the pancreatic imaging is imperfect, and hence may not be diagnostic of serous cystadenoma.

In our patient, the non-specific clinical and laboratory investigations, combined with a lobulated, multicystic pancreatic mass with focal calcification upon radiological imaging may lead to an erroneous primary diagnosis of serous cystadenoma. We conclude that pancreatic tuberculosis should be considered in the differential diagnosis of solitary pancreatic cystic lesions, even in healthy patients.

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CASE REPORT

Nodular liver lesions involving multiple myeloma: A case report and literature review

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Abstract

We report a case of a 62-year old woman admitted to our hospital for multiple nodular metastatic liver lesions found by ultrasonography in a regular medical examination. Routine laboratory tests were normal. PET-CT showed multiple bone lesions and nodular liver lesions. Liver biopsy revealed nodular infiltration of multiple myeloma with positive staining of kappa light chain. Further investigation of bone marrow aspiration, immunofixation and immunoelectrophoresis of serum protein, urine test for Bence-Jones protein, β_2-microglobulin in serum and urine confirmed the diagnosis. The patient also coinfected with hepatitis C virus (HCV). With six cycles of chemotherapy with VAD schedule, she achieved complete remission. In this report, a literature review of liver lesions involving multiple myeloma is also provided.

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Key words: Nodular lesions; Liver; Multiple myeloma; IgA kappa light chain; Hepatitis C virus

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INTRODUCTION

Multiple myeloma (MM) is a malignant plasma cell disorder characterized by plasma cell infiltration of the bone marrow and overproduction of immunoglobulin or light chains. Although plasma cell infiltration of the liver occurs in 40% cases of MM[1], most of them are diffused infiltration and usually reported as autopsy findings. Nodular liver lesions involving MM is a rare condition. We present a case of hepatic nodular lesions identified to be MM by liver biopsy.

CASE REPORT

A 62-year old woman was admitted to our hospital for multiple nodular metastatic liver lesions found by ultrasonography in a regular medical examination. She had low back pain for four months, which was diagnosed as lumbar vertebral osteoarthrosis, and treated with Votalins, but did not remit. She had thrombocytopenic purpura 20 years ago and received blood transfusion during choledocholithotomy for bile duct stone 10 years ago.

On physical examination, the only positive sign was tenderness in processus spinalis L3-S1 and paravertebral area.

Laboratory tests on admission showed 102 g/L hemoglobin, 5.35 × 10^9/L white blood cells, 242 × 10^9/L platelets, 93 mm/h erythrocyte sedimentation rate (ESR). C-reactive protein, liver function test, lactate dehydrogenase (LDH), electrolytes, glucose, fat, and renal function tests were all normal. Prothrombin time (PT) was 12.9 s, prothrombin activity (PTA) was 85.8%. Serum markers for hepatitis B were negative, while hepatitis C antibody was positive, and HCVRNA was 1.47 × 10^5 IU/L.

Abdominal computed tomography (CT) showed multiple metastatic space-occupying lesions of the liver and destruction of multiple lumbar vertebral bodies. F-18 fluorodeoxyglucose (FDG) positron emission tomography (PET)/CT imaging demonstrated multiple osteolytic bone lesions including vertebrae of cervical,

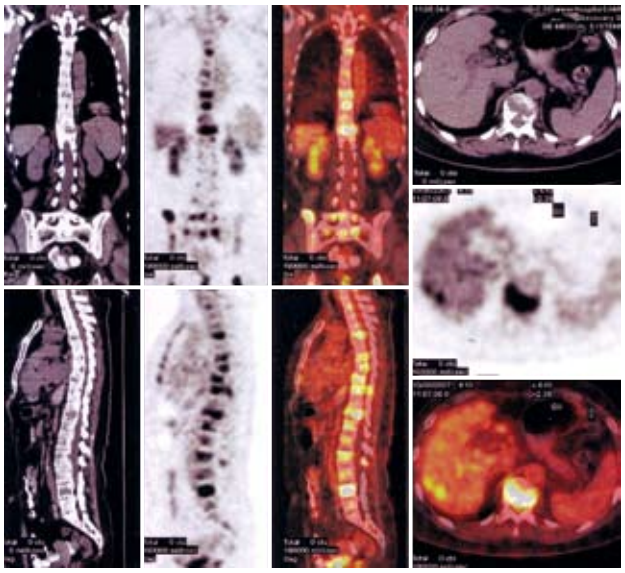

Figure 1 Multiple osteolytic bone lesions and nodular liver lesions (PET-CT).

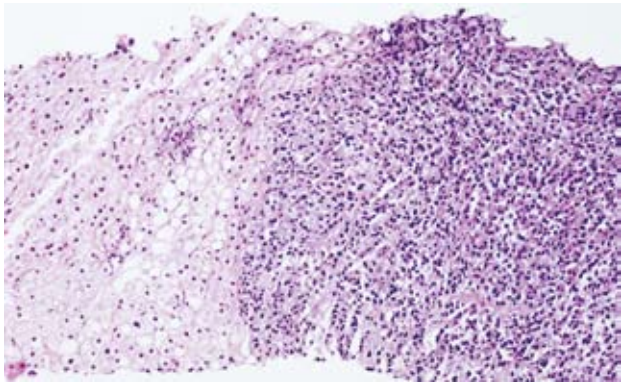

Figure 2 Nodular plasma cell infiltration in liver: myeloma on the right, liver tissue on the left and steatosis in the middle (HE-20 ×).

thoracic, lumbar and sacral, and skull, mandible, right humerus, scapula, left clavicle, sternum, ribs, and pelvic bones as well as several hypermetabolic areas in liver, indicating metastatic tumors (Figure 1).

Gastroentestinal endoscopy revealed no primary tumors. Fine-needle liver biopsy under ultrasonography guidance was performed, showing focal lesions of plasma-cell myeloma (Figures 2 and 3), which was positive for immunohistochemistry staining of kappa light chain (Figure 4). Congo red staining showed slight amyloid substance deposition in the sinusoidal area. Histopathologic examination of the right iliac crest confirmed the diagnosis of multiple myeloma (Figure 5). Plasmacytosis (28.5%) showed immature plasmablasts with prominent nucleoli. The number of residual normal hemopoeitic cells was markedly reduced.

The hallmark of MM is the detection of monoclonal and M protein in blood and/or urine, produced by abnormal plasma cells. Immunofixation and immunoelectrophoresis confirmed the presence of a broad monoclonal band serum protein in β globulin regions. The band type was identified as IgA kappa at a concentration of 1770 mg/dL. Urine test for Bence-Jones protein was positive. β_2-microglobulin in serum and urine were 3.71 μg/mL (reference range 1.17-2.29) and > 8 mg/24 h (reference range 0-0.637), respectively. Creatinine clearance rate was 54.8 mL/min (reference range 75-115). Finally, the patient was diagnosed as multiple myeloma, IgA kappa light chain, grade Ⅱ, with nodular liver involvement, and coinfection with hepatitis C virus (HCV).

She started systemic chemotherapy with Bortezomib, vincristine, doxorubicin along with oral dexamethasone,

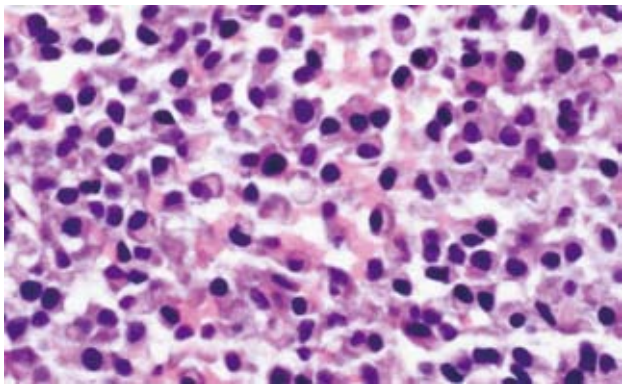

Figure 3 Plasma cell infiltration in liver (HE-60 ×).

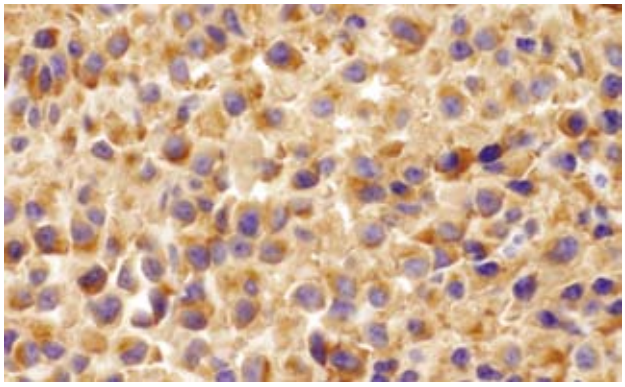

Figure 4 Positive staining of kappa light chain by immunohistochemistry in liver (60 ×).

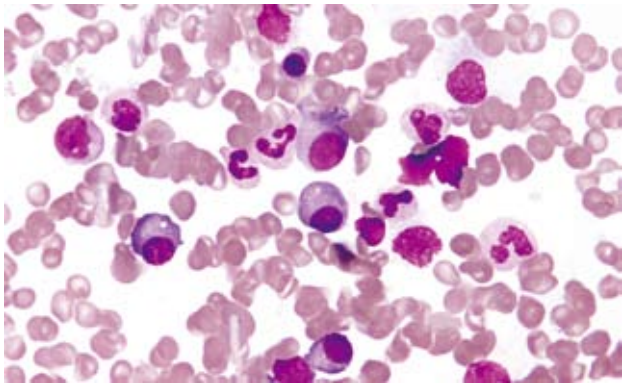

Figure 5 Plasmacytosis in bone marrow (HE-60 ×).

and achieved complete remission. Bone marrow aspiration showed that the number of plasma cells was less than 1%. Immunofixation and immunoelectrophoresis detected no monoclonal band serum protein. Urine Bence-Jones protein was negative. β_2-microglobulin in serum and urine decreased to 2.2 μg/mL and 0.857 mg/24 h, respectively. CT scan of the abdomen six months after therapy showed no focal lesions in liver and SPECT did not find new lesions in bone.

DISCUSSION

Nodular liver lesion has been proved to be MM by liver biopsy, which is a clonal B-lymphocyte neoplasm of terminally differentiated plasma cells. Recurrent bacterial infection, anemia, osteolytic lesions and renal insufficiency are the most common clinical features of MM. Our patient had a history of low back pain for four months, but her uncharacterized laboratory tests and image examination did not show enough evidence of MM at the beginning. Then, metastatic nodular liver lesions were incidentally found during a regular medical examination. From a clinical point of view, metastatic liver lesions might have led us to think of metastasis of primary adenocarcinoma from gastrointestinal tract, breast, and adrenal glands. Unexpectedly, fine-needle aspiration showed nodular plasma-cell myeloma in liver. PET-CT scan showed characteristic multiple liver lesions and many skeletoles, but no other organ infiltration.

When MM affects the liver, diffused (sinusoidal, portal, mixed) and nodular patterns of microscopic plasma cell infiltration have been described[1]. Diffused rather than nodular hepatic infiltration is the predominant pattern of liver involvement. Perez-Soler *et al*[2] reported 128 patients with MM, histologic findings were available in the liver of 21 patients, and diffuse plasma cell infiltration of the liver was observed in 10 patients, while no case of nodular liver infiltration was observed. In a database of 2584 patients with MM[3], liver involvement could be found as masses or macroscopic nodules only in 9 cases. However, in a retrospective study on 52 autopsied cases with MM[4], hepatic invasion can be observed in 15 patients (28.8%) with circumscribed tumor nodules in 7 cases (13.4%) and diffuse tumor involvement in 8 cases (15.4%). In fact, most nodular liver lesions involving MM have been reported in single cases[5-9].

Myeloma cells proliferate in bone marrow and circulate through bloodstream. Like benign plasma cells, they circulate through lymphatics and the reticuloendothelial system. Hence spleen, liver or lymph node infiltrations are common. Amyloidosis and myeloid metaplasia are also histological changes leading to clinical presentations. Liver lesions involving MM may present with hepatomegaly, jaundice, ascites, fulmnant liver failure, or are totally asymptomatic incidentally found by autopsy or image examination. It was reported[1] hepatolmegaly can be found in 58% cases of MM and plasma cell infiltration of the liver is confirmed in 40% cases of MM. However, in all hematological malignancies[10], liver involvement is found in 32% of cases of myeloma, 80%-100% in chronic leukemia and myeoproliferative diseases, 60%-70% in acute leukemia, and 50%-60% in non-Hodgkin's lymphoma.

The correlation between paraprotein type and incidence of extraosseous tumor in patients with myeloma still remains unclear[11]. Edward *et al*[12] suggested that extraosseous involvement may be more prominent in patients with IgA myeloma. Oshima *et al*[4] found that extraosseous involvement of the liver is more frequent in IgA myeloma than in other types of myeloma, although the reason is unknown. In our case, IgA kappa light chain deposited in the liver sinus was confirmed by immunohistochemistry.

Interestingly, our patient was also coinfected with HCV. HCV is lymphotropic and may replicate in lymphocytes and hepatocytes. It was reported that multiple myelomas may develop in a patient with chronic hepatitis C[13]. The prevalence of HCV infection in patients with MM is 6.5%[14]. In contrast to HBV exacerbation, HCV rarely causes fulminant hepatitis, but less is known about the exacerbation of chronic HCV infection in patients with hematological malignancies undergoing chemotherapy and corticosteroid treatment. In our case, her liver function test was normal, image examination showed no signs of liver fibrosis, and liver biopsy revealed no significant hepatitis or liver fibrosis. However, close monitoring and management of coinfection with HCV are necessary and should be in coordination with therapy for MM.

In summary, we described a case of multiple space-occupying lesions in liver unexpectedly proved to be multiple myeloma. Liver lesions involving MM should be differentially diagnosed from other diseases.

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LETTERS TO THE EDITOR

Triptolide and management of systemic malignancies besides pancreatic carcinomas

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Abstract

The recent article by Zhou *et al* was highly interesting and thought provoking. The authors have clearly shown that triptolide administration is associated with up-regulation of the *Bax* gene, resulting in an attenuating effect on cell growth in gastrointestinal malignancies such as pancreatic carcinomas. The article by Zhou *et al* is all the more important because it highlights the rapidly increasing role of triplodide in the management of systemic malignancies. For instance, triptolide acts on the PI3K/Akt/NF-κB pathway, thereby enhancing apoptosis secondary to the administration of bortezomib in multiple myeloma cells. Similar synergisms are seen when triptolide is administered along with 5-fluoruracil for the management of colonic carcinomas. Similarly, triptolide causes down-regulation of the *Bcl-2* gene, resulting in control of cell growth in tumors, such as glioblastoma multiformes.

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Key words: Triptolide; *Bax* gene; *Bcl-2* gene; SDF-1/CXCR4 pathway; Acute T lymphocytic leukemias

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TO THE EDITOR

The recent article by Zhou *et al*[1] was highly interesting and thought provoking. The authors have clearly shown that triptolide administration is associated with up-regulation of the *Bax* gene, resulting in an attenuating effect on cell growth in gastrointestinal malignancies, such as pancreatic carcinomas[1]. The article by Zhou *et al*[1] is all the more important because it highlights the rapidly increasing role of triplodide in the management of systemic malignancies.

For instance, triptolide acts on the PI3K/Akt/NF-κB pathway thereby enhancing apoptosis secondary to the administration of bortezomib in multiple myeloma cells[2]. Similar synergisms are seen when triptolide is administered along with 5-fluoruracil for the management of colonic carcinomas[3]. Similarly, triptolide causes down-regulation of the *Bcl-2* gene resulting in control of cell growth in tumors, such as glioblastoma multiformes[4]. In fact, triptolide, when combined with ionizing radiation in the therapy of pancreatic carcinomas, decreases cell survival in these tumors by almost 21%[5].

Triptolide also inhibits the SDF-1/CXCR4 pathway and thereby has an attenuating effect on lymphoid metastatic, as well as proliferative activity in non-Hodgkin lymphoma cell lines[6]. Similarly, triptolide demonstrates anti-proliferative effects in other hematological malignancies, such as acute myeloid leukemia. In fact, the anti-carcinogenic effects of triptolide in malignancies, such as acute myeloid leukemia are markedly enhanced by other agents such as AraC[7]. Recent studies also confirm that triptolide has a negative effect on proliferation in acute T lymphocytic leukemia[8]. These anti-carcinogenic functions of triptolide are in part secondary to its anti- angiogenic properties[9].

More recently, Xu *et al*[10] have developed polymeric micelles of triptolide which appear to demonstrate anti-carcinogenic properties without affecting host immunity. These recent developments further highlights the immense therapeutic potential of triptiolide and the need for further research to fully assess its anti-carcinogenic potential.

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Meetings

Events Calendar 2009

January 12-15, 2009
Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

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

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

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

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

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

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

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

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

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

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

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

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

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

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

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

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

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

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

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

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

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

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
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Acknowledgments

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

Patent (list all authors)

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

Statistical data

Write 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 υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc*.
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