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Contents

Contents

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Contents

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EDITORIAL

Eradication of *H pylori* for the prevention of gastric cancer

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Abstract

Infection with *H pylori* is the most important known etiological factor associated with gastric cancer. While colonization of the gastric mucosa with *H pylori* results in active and chronic gastritis in virtually all individuals infected, the likelihood of developing gastric cancer depends on environmental, bacterial virulence and host specific factors. The majority of all gastric cancer cases are attributable to *H pylori* infection and therefore theoretically preventable. There is evidence from animal models that eradication of *H pylori* at an early time point can prevent gastric cancer development. However, randomized clinical trials exploring the prophylactic effect of *H pylori* eradication on the incidence of gastric cancer in humans remain sparse and have yielded conflicting results. Better markers for the identification of patients at risk for *H pylori* induced gastric malignancy are needed to allow the development of a more efficient public eradication strategy. Meanwhile, screening and treatment of *H pylori* in first-degree relatives of gastric cancer patients as well as certain high-risk populations might be beneficial.

Key words: Gastric cancer; *H pylori*; Eradication

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INTRODUCTION

Despite decreasing incidence and mortality rates, gastric cancer remains the second most frequent malignancy worldwide, with the majority of cases diagnosed at an advanced stage[1]. A number of environmental factors, e.g. diets high in salt and N-nitrosamines and low in fruits and vegetables have been shown to contribute to gastric cancer development[2]. Furthermore, it is now well recognized that chronic infection with *H pylori* is tightly associated with the development of gastric cancer, primarily noncardiac gastric cancer. The clinical course of *H pylori* infection is highly variable and the likelihood of developing gastric cancer is determined by both microbial and host factors (Figure 1). Based on the large number of experimental and epidemiological studies, it seems reasonable to conclude that the eradication of *H pylori* should prevent gastric cancer. However, convincing results from clinical trials are not yet available. Hence, current clinical decision-making has to be based on indirect evidence: data from animal models and studies supporting the beneficial effect of eradication on the development of gastric cancer precursor lesions[3]. This article reviews the existing evidence that *H pylori* eradication prevents gastric cancer with a highlight on recent publications relevant for the clinician.

PATHOGENESIS AND EPIDEMIOLOGY

Pathogenesis

According to Correa's model, gastric cancer development is a multistep process where the gastric mucosa is slowly transformed from normal epithelium to chronic gastritis, to multifocal atrophy, to intestinal metaplasia of various degrees, to dysplasia and finally to invasive cancer[4]. However, this sequence of events does not precede diffuse type gastric cancer and has even been debated for the intestinal type[5] since less than 10% of patients with these lesions ultimately develop gastric cancer[6]. Most *H pylori* infected individuals show antral predominant gastritis, which predisposes them to duodenal ulcers, but rarely causes gastric cancer. On the contrary, patients with corpus-predominant gastritis are likely to develop gastric ulcers, gastric atrophy, intestinal metaplasia and eventually gastric cancer. Our group, among others, has found that the pattern and the morphological distribution of gastritis correlate strongly with the gastric cancer risk[7,8]. We showed that the expression of *H pylori* associated gastritis in patients with gastric cancer is high in the corpus and is frequently associated with intestinal metaplasia and atrophy[9]. Based on these findings we developed a gastric carcinoma risk index, which evaluates grade and activity of corpus-dominant *H pylori* gastritis as well as the occurrence of intestinal metaplasia in the antrum or corpus to determine a patient's risk for developing gastric carcinoma[10]. In a subsequent case control study, the gastric carcinoma risk index had a sensitivity of 93% and a specificity of 85% for diagnosing individuals with gastric carcinoma[11].

Epidemiology

Infection with *H pylori* occurs worldwide, but the prevalence varies greatly among countries and among different populations within the same country[12]. The overall prevalence of *H pylori* infection is closely linked to current socioeconomic conditions[13]. Although the incidence of the infection in industrialized countries has decreased substantially over recent decades, it will remain endemic for at least another century, unless intervention occurs[14]. In the early 1990s a series of prospective case control studies[15-18] demonstrated a close link between *H pylori* infection and gastric cancer, which prompted the World Health Organization to announce the bacterium a class I (definite) carcinogen in 1994. Since then data from various studies have accumulated that further strengthen the association between *H pylori* infection and gastric cancer. One of the most compelling studies was conducted in Japan, where Uemura *et al*[19] prospectively followed 1526 patients over a period of 7.8 years. A total of 2.9 percent of *H pylori* infected individuals developed gastric cancer compared to none in the *H pylori* negative control group. Among individuals with *H pylori* infection, those with severe gastric atrophy (odds ratio: 4.9), corpus-predominant gastritis (odds ratio: 34.5) and intestinal metaplasia were at significantly higher risk for gastric cancer.

According to most retrospective, cohort and case control studies, the overall odds ratio for *H pylori* infection and gastric cancer is around two to six[19-23]. However, these numbers are likely to represent a gross underestimation of the real risk. Among the confounding factors that make risk appear lower in most studies are the long latency between the initiation of the carcinogenetic process and the clinical occurrence of cancer as well as the inclusion of individuals with antral predominant/duodenal ulcer phenotype[24]. If selection of patients and methodology is optimized, the odds ratio for *H pylori* infected individuals may increase to a factor of around 20[25-27].

RISK FACTORS

Bacterial virulence factors

H pylori displays a considerable amount of genetic variation. Even strains within an individual host commonly change over the course of the infection[28,29]. A number of bacterial virulence factors have been discovered that influence disease outcome in infected individuals. The majority of *H pylori* strains express and secrete VacA, a vacuolating cytotoxin, which is inserted into the gastric epithelial-cell and mitochondrial membranes, possibly providing the bacterium with nutrients and inducing apoptosis of the host cell[12,30]. VacA has also been found to modulate the host immune system *via* T-cell inhibition[31,32]. Studies indicate that expression of VacA increases bacterial fitness and in some western countries VacA *s1* and VacA *m1* genotypes are associated with more severe forms of gastritis, atrophy, intestinal metaplasia and perhaps gastric cancer[33-36]. Another major focus of research is the analysis of the *cag* pathogenicity island (*cag PAI*), a genomic fragment comprising 31 genes that support the translocation of the 120-kD CagA protein into the gastric epithelial cell[37,38]. CagA has been shown to induce cytokine production along with a growth factor-like response in the host cell and to disrupt the junction-mediated gastric epithelial cell barrier function[39,40]. In western countries, patients carrying CagA+ *H pylori* strains are more likely to develop adenocarcinomas of the distal stomach than patients infected with CagA- strains[41]. In particular, one recent meta-analysis of case-control studies concluded that infection with CagA+ strains increases the risk over *H pylori* infection alone[42]. However, similar findings are not reported from Asia, where about 95% of all infected individuals carry CagA+ strains[3,43,44].

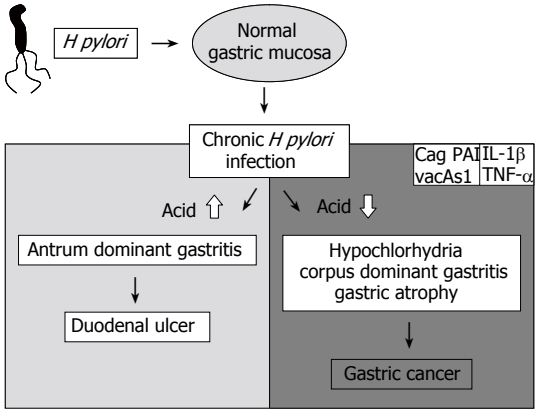

Figure 1 Variable course of *H pylori* infection.

Host genetic factors

H pylori leads to inflammation of the gastric mucosa in virtually all infected individuals. However, most *H pylori* infected humans do not develop gastric cancer even if they are infected with so-called more virulent strains, indicating that host factors play a crucial role. The fact that first-degree relatives of gastric cancer patients have a significantly increased risk for developing gastric cancer compared to patients without a family history further emphasizes the importance of genetic factors. For example, our group found some important gastric cancer related genes to be more prevalent in the gastric mucosa of first-degree relatives[45-49].

The infection with *H pylori* triggers an extensive systemic and local inflammatory response. Gastric epithelial cells respond by producing enhanced levels of interleukin-1β, interleukin-2, interleukin-6, interleukin-8 and tumor-necrosis-factor-α[50-52]. El-Omar and co-workers were the first to show that patients with certain Interleukin-1 gene cluster polymorphisms, which lead to enhanced production of the proinflammatory cytokine IL-1β are at increased risk for *H pylori* induced hypochlorhydria and gastric cancer[53]. Further studies found that proinflammatory polymorphisms of the IL-1 receptor antagonist, tumor necrosis factor-α and IL-10 are also associated with an increased risk for the development of noncardia gastric adenocarcinoma[54,55]. Interestingly, the combination of pro-inflammatory polymorphisms in the interleukin-1β gene and infection with more virulent *H pylori* strains seems to increase the gastric cancer risk even more[56]. Most of the important studies exploring host

Table 1 Gastric cancer prevention studies

Study	Design	Follow-up	Patients	Treatment	Outcome
Uemura *et al* 1997	Nonrandomized intervention trial	2 yr	132 Japanese patients with endoscopically removed early stage gastric cancer and *H pylori* infection	*H pylori* eradication therapy or no treatment	Reduced rate of gastric cancer development after eradication of *H pylori*
Saito *et al* 2000	Nonrandomized intervention trial	2 yr	64 Japanese patients with gastric adenoma and *H pylori* infection	*H pylori* eradication therapy or no treatment	Reduced rate of metachronous gastric cancer development after eradication of *H pylori*
Correa *et al* 2000	Prospective, randomized, placebo controlled trial	6 yr	852 individuals from a high risk region in Colombia with *H pylori* infection and precancerous lesions	*H pylori* eradication therapy and/or ascorbic acid/beta-carotene or placebo	Significant increase in the rate of regression of precursor conditions after cure of *H pylori* and/or treatment with dietary supplements
Wong *et al* 2004	Prospective, randomized, placebo controlled trial	8 yr	1630 individuals from a high risk region in China with *H pylori* infection; with or without precancerous lesions	*H pylori* eradication therapy or placebo	Significant reduction in gastric cancer risk after cure of *H pylori* only for patients without precancerous conditions
Take *et al* 2005	Nonrandomized intervention trial	3.4 yr	1342 Japanese patients with peptic ulcer disease	*H pylori* eradication therapy	Significant increase in gastric cancer risk for patients with persistent *H pylori* infection

genetics were performed in Caucasian populations and still need to be confirmed in other ethnic groups[3]. However, there is emerging evidence that similar associations can be found in Asian populations. For example, one study from Japan showed that proinflammatory IL-1β polymorphisms in *H pylori* infected Japanese individuals are significantly associated with hypochlorhydria and atrophic gastritis[57]. Recent data by Goto *et al*[58] also indicate that a common polymorphism in the coding gene for SHP-2 that interacts with the CagA protein can increase the risk for gastric atrophy in Japanese patients infected with CagA+ *H pylori* strains. The authors speculate that this might explain why only a proportion of CagA+ individuals develop gastric atrophy even though this strain is almost universal in Asian countries.

IN VIVO STUDIES

Animal models

A number of animal models have been developed to study the mechanisms by which *H pylori* induces gastric carcinogenesis. Using the Mongolian gerbil model, several studies provided evidence that *H pylori* infection is in fact a potent carcinogen and able to induce gastric cancer by itself[59-62]. The studies by Watanabe *et al*[59] and Honda *et al*[60] found that 37% and 40% of infected animals developed well-differentiated intestinal adenocarcinomas 62 and 72 wk after inoculation of the bacterium. Both studies used *cagA* and *vacA* positive *H pylori* strains for infection of the animals. The risk of gastric carcinogenesis in Mongolian gerbils increases significantly through combination of *H pylori* infection with other known carcinogens such as N-methyl-N-nitrosourea (NMU) and N-methyl-N-nitro-N-nitrosoguanidine (MNNG)[63-65]. Studies using *H pylori* or *H. felis* infected mice found that the gastric cancer development is strongly determined by host specific factors, for example specific patterns of immune response. Some mouse strains develop a vigorous Th-1 response to the infection while others have a predominant Th-2 immune response and seem to be more resistant to mucosal damage. Those with the strong TH-1 response continue to develop atrophy, metaplasia and eventually invasive cancer in a gender specific manner[66].

There is evidence from animal models, that eradication of *H pylori* is able to prevent gastric carcinogenesis. The incidence of gastric adenocarcinoma in nitrosamine administered Mongolian gerbils with *H pylori* infection was significantly lower in animals receiving *H pylori* eradication[67,68]. Mouse models have also provided important evidence of beneficial effects from the administration of anti-inflammatory drugs, where atrophy and metaplasia have been reversed, in some cases completely[69].

Clinical studies

Cure of *H pylori* infection results in several physiologic effects that are likely to reduce gastric cancer risk. These include reduction in cell turnover, elimination of DNA damage by a reduction of reactive oxygen species, increased gastric acid secretory capacity and restoration of ascorbic acid secretion into the gastric juice[1,70,71]. However, evidence from well-designed clinical studies supporting the cancer protective effect of *H pylori* eradication remains sparse (Table 1). Among the first clinical data to support the hypothesis that *H pylori* eradication is able to prevent gastric cancer development were case-control studies from Sweden on patients undergoing hip replacement procedures. Akre *et al*[72] showed that significantly reduced rates of gastric cancer occurred in such patients who frequently receive high doses of prophylactic antibiotics, incidentally eradicating *H pylori* infection. As discussed earlier in this review, the study by Uemura *et al*[19] provides some of the strongest evidence for the causative role of *H pylori* infection in gastric cancer development. Here, gastric cancer developed in 2.9% of all *H pylori* infected patients compared with 0% of those without infection. Notably,

no case of cancer developed in a subgroup of 253 *H pylori* infected patients who received eradication therapy at an early time point after enrollment in the study. The same group of investigators found that eradication of *H pylori* was able to prevent relapse after endoscopic resection of early stage gastric cancer[73]. Another study by Saito *et al*[74] showed that *H pylori* eradication had a favorable impact on gastric cancer development in patients with gastric adenoma. More recently, Take *et al*[75] published the results from a large prospective Japanese intervention trial. The authors endoscopically followed 1342 patients with peptic ulcer disease for a mean period of 3.4 years. All patients initially received *H pylori* eradication therapy. The risk of developing gastric cancer was significantly higher in the group of patients who failed eradication therapy compared to those who were cured for the infection.

The first prospective randomized controlled study to examine the effect of *H pylori* eradication on gastric cancer development was published by Wong *et al*[76] in 2004. The authors randomized 1630 individuals from a high-risk region in China with confirmed *H pylori* infection to eradication therapy or placebo. After a follow-up period of 7.5 years, they found no difference in gastric cancer incidence between those receiving *H pylori* eradication therapy and those who were not given treatment (7 *vs* 11 cases, $P = 0.33$). However, further subgroup analysis of the data demonstrated a significant benefit ($P = 0.02$) from eradication therapy in patients without baseline intestinal metaplasia at the time of study enrollment.

Unfortunately, several international prospective randomized controlled trials, designed to evaluate the long-term effect of *H pylori* eradication on gastric cancer development had to be abandoned. For example, the PRISMA study[77], initiated in 1998 by our group to test the effect of *H pylori* eradication therapy in a high-risk population in Germany and Austria was discontinued due to insufficient recruiting. As might be expected, most eligible patients for those studies are not willing to enter the placebo arm after the nature of such a trial has been explained to them. Apart from ethical issues, the required follow-up time of 10 to 20 years for these trials remains an additional problem. A growing number of studies are therefore using surrogate markers for gastric cancer development, namely gastric atrophy and intestinal metaplasia as primary study endpoints.

There is consistent evidence that *H pylori* eradication cures gastritis and numerous studies have shown that atrophy and metaplasia do not progress in patients after *H pylori* eradication compared to control groups who remain *H pylori* positive[78-86]. However, many of the available studies addressing the reversibility of gastric atrophy and intestinal metaplasia have yielded conflicting and inconsistent results, possibly because most of them are nonrandomized, not controlled, have short follow-up periods and only include small numbers of patients[1,3]. One of the few randomized controlled trials for the prevention of gastric dysplasia was conducted by Correa *et al*[87] in 2000. The authors found significant regression of gastric atrophy and intestinal metaplasia after *H pylori* eradication alone and in combination with β-carotene and ascorbic acid. Sung *et al*[88] prospectively followed a total of 587 *H pylori* infected patients, randomized to receive either eradication therapy or placebo, endoscopically for one year. Decrease in acute and chronic gastritis was significantly more frequent after *H pylori* eradication, but after the relatively short follow-up period, changes in intestinal metaplasia were similar between the two groups. The majority of available studies suggest, however, that regression of atrophic gastritis and, to a lesser extent, intestinal metaplasia can occur at least in a subset of patients with sufficient follow-up[3,89].

In conclusion, there is little randomized controlled trial evidence to suggest that *H pylori* eradication decreases the risk of gastric cancer development. However, regression of gastric cancer precursor lesions may occur in some patients. At present, there are no markers that help to predict such a response in the individual patient. Therefore, eradication at the earliest possible time point in the disease process seems favorable. The optimal age for testing of *H pylori* infection still needs to be determined but available data suggest that eradication at a younger age might be a more favorable approach. Future research has to focus on identification of host and bacterial specific markers that will help to predict the development of gastric cancer in the *H pylori* infected individuals. Better identification of individuals at high risk for gastric cancer will allow for more effective prevention and eradication strategies. Meanwhile, screening and treatment of *H pylori* in first-degree relatives of gastric cancer patients as well as certain high-risk populations might be beneficial.

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EDITORIAL

Endoscopic submucosal dissection for stomach neoplasms

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Abstract

Recent advances in techniques of therapeutic endoscopy for stomach neoplasms are rapidly achieved. One of the major topics in this field is endoscopic submucosal dissection (ESD). ESD is a new endoscopic technique using cutting devices to remove the tumor by the following three steps: injecting fluid into the submucosa to elevate the tumor from the muscle layer, pre-cutting the surrounding mucosa of the tumor, and dissecting the connective tissue of the submucosa beneath the tumor. So the tumors are resectable in an *en bloc* fashion, regardless of the size, shape, coexisting ulcer, and location. Indication for ESD is strictly confined by two aspects: the possibility of nodal metastases and technical difficulty, which depends on the operators. Although long-term outcome data are still lacking, short-term outcomes of ESD are extremely favourable and laparotomy with gastrectomy is replaced with ESD in some parts of therapeutic strategy for early gastric cancer.

Key words: Therapeutic endoscopy; Endoscopic submucosal dissection; Stomach neoplasia; Early cancer; Node-negative tumor

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INTRODUCTION

Endoscopic resection of stomach neoplasms has been originated from the development of a polypectomy technique using the high-frequency current to gastric polyps in 1968[1,2], and it has become popular as endoscopic mucosal resection (EMR) since the birth of a strip biopsy method in 1984[3] and a cap technique in 1993[4]. Endoscopic submucosal dissection (ESD) technique is a new endoscopic treatment using cutting devices, which has developed from one of the EMR techniques, namely endoscopic resection after local injection of a solution of hypertonic saline-epinephrine (ERHSE)[5]. ESD consists of the following three steps: injecting fluid into the submucosa to elevate the tumor, pre-cutting the surrounding mucosa of the tumor, and dissecting the connective tissue of the submucosa beneath the tumor[6-10]. Major advantages of this technique in comparison with conventional EMR are as follows. The resected size and shape can be controlled, *en bloc* resection is possible even in a large tumor, and tumors with ulcerative findings are also resectable. So this technique can be applied to the resection of complex tumors such as large tumors, ulcerative non-lifting tumors, and recurrent tumors. The disadvantages of this technique are requirement of 2 or more assistants, time-consuming, much more bleeding and a little higher perforation rate than conventional EMR[11]. It is still controversial whether the esophageal or colorectal neoplasms in an early stage should be resected in an *en bloc* fashion by using ESD, considering the technical difficulty, associated risks, and favorable outcomes by conventional EMR[12-16]. However, in case of the stomach neoplasms, especially when large or ulcerative tumors are targeted as the subjects of endoscopic resection, necessity of *en bloc* resection is emphasized, because multi-fragmental resection causes insufficient histological evaluation and local recurrence of multi-fragmental resection becomes significantly higher than that of *en bloc* resection[17]. So in Japan, ESD is now gaining acceptance as the standard endoscopic resection techniques for stomach neoplasms in an early stage, especially for large or ulcerative tumors.

INDICATION FOR ENDOSCOPIC SUBMUCOSAL DISSECTION

Although institutional differences in indications for endoscopic resection have existed for a long time, empirical indication for conventional EMR is differentiated-type of mucosal cancers without ulcerative findings, with ≤ 2 cm in size if elevated or ≤ 1 cm in size if depressed or flat[18]. The Japanese Gastric Cancer Association issued their gastric cancer treatment guidelines in 2001, showing that differentiated-type of mucosal cancers without ulcerative findings, with ≤ 2 cm in size, regardless of the tumor morphology are practically indicated for endoscopic resection[19]. These criteria are determined by considering two aspects: the conditions free of lymph node metastasis and

Table 1 Frequency of lymph node metastases in early gastric cancer[20]

Criteria	Frequency (No. with metastasis/total number)	95% CI
Intramucosal cancer differentiated adenocarcinoma, no lymphatic vascular invasion, irrespective of ulcer findings, tumor ≤ 3 cm	0/1230	0-0.3%
Intramucosal cancer differentiated adenocarcinoma, no lymphatic vascular invasion, without ulcer findings, irrespective of tumor size	0/929	0-0.4%
Intramucosal cancer undifferentiated adenocarcinoma, no lymphatic vascular invasion, without ulcer findings, tumor ≤ 2 cm	0/141	0-2.6%
Cancer with minute submucosal penetration (≤ 500 μm) differentiated adenocarcinoma, no lymphatic vascular invasion, irrespective of ulcer findings, tumor ≤ 3 cm	0/145	0-2.5%

the probability of successful *en bloc* resection. If the technical problems are overcome, indication could be expanded for all tumors which have been elucidated as node-negative tumors in clinical trials (Table 1)[20].

TECHNIQUES OF ENDOSCOPIC SUBMUCOSAL DISSECTION

ESD requires special cutting knives, such as a needle knife[5], an insulation-tipped electrosurgical (IT) knife[6,21-24], a hook knife[25,26], a flex knife[8-10], and a triangle-tip (TT) knife[27,28], or special devices such as a small-caliber tip transparent (ST) hood[29] (Figure 1). As another approach to successful ESD, investigations of submucosal injection solutions have been actively done. It was reported that a hyaluronic acid solution makes a better long-lasting submucosal cushion without tissue damage than other available solutions[29-33]. As a further improvement of hyaluronic acid solution, usefulness of a mixture of high-molecular-weight hyaluronic acid, glycerin, and sugar has also been reported[34,35]. A representative case of ESD procedure using a flex-knife and a mixture of high-molecular-weight hyaluronic acid, glycerin, and sugar is shown in Figure 2[36].

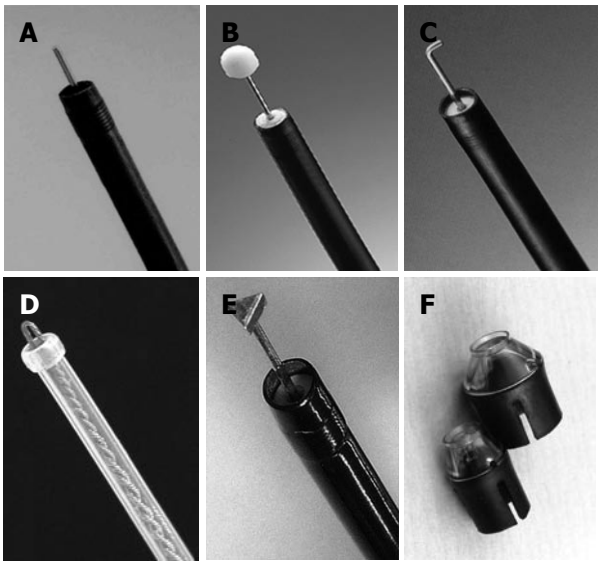

Figure 1 Devices for ESD. **A**: Needle knife (KD-1L-1, Olympus, Tokyo, Japan); **B**: IT (KD-610L, Olympus, Tokyo, Japan); **C**: Hook knife (KD-620LR, Olympus, Tokyo, Japan); **D**: Flex knife (KD-630L, Olympus, Tokyo, Japan); **E**: TT knife (KD-640L, Olympus, Tokyo, Japan); **F**: ST hood (DH-15GR, 15CR, Fujinon Toshiba ES Systems, Tokyo, Japan).

Marking around the tumor

After chromoendoscopy using 0.25% indigo carmine to clarify the border of the tumor, circumferential markings are made by using a flex-knife, which is set to about 1 mm in length of the knife, at about 5 mm outside of the tumor with 2-mm intervals between each marking.

Submucosal injection

Solutions prepared for submucosal injection are a 10% glycerine with 0.9% NaCl plus 5% fructose solution (glyceol, Chugai Pharmaceutical co., Tokyo Japan) alone for small distal gastric tumors without ulcer findings and a mixture of glyceol and 1% 1900 ku hyaluronic acid preparation (suvenyl, Chugai Pharmaceutical co. Tokyo Japan) for complex or proximal gastric tumors. The mixing ratio of glyceol and suvenyl is 7:1 for complex or proximal gastric tumors. A small amount of epinephrine to make a concentration of 0.0005% is added to obtain vasoconstriction for hemostasis and indigo carmine is also added to find out the seeping area of the submucosal injection solution as operators' preference. Injection of these solutions is performed into the submucosal layer just outside the markings where mucosal incision intends to be made at first. The volume of injection is about 2 mL once, and injection is repeated several times before starting mucosal incision until the targeted area is lifted enough. After exposure of the submucosal layer, injection is applicable from the exposed submucosal layer to lift up the submucosal layer intended to be cut.

Mucosal incision

After the tumor is lifted, mucosa outside the markings is incised circumferentially by using the same flex-knife, which is set to about 2 mm in length of the knife. The starting point for cutting is principally a distal part from the mouth. Retroflex position of the endoscope is usually used if applicable when the distal part of the tumor is cut.

Submucosal dissection

Small tumors can be resected by an electrosurgical snare only after mucosal incision around the markings without submucosal dissection. However, large tumors and tumors with ulcer findings or located in a tortuous area cannot be resected by an electrosurgical snare, dissecting the submucosa completely is thus needed. A flex-knife, which is set to about only 1 or 2 mm in length of the knife is also

Table 2 Outcomes of ESD

Techniques	En bloc resection rate %		Local recurrent rate %	Complication rate %	
	≤ 20 mm	> 21 mm		Bleeding	Perforation
ESD with IT knife[42]	95 (686/719)	86 (271/314)	NA	6 (59/945)	4 (35/945)
ESD with the tip of an electrosurgical snare (thin type)/a flex knife[8]	95 (56/59)		NA	1.7 (1/59)	3.4 (2/59)
ESD with sodium hyaluronate and small-caliber-tip transparent hood[43]	100 (37/37)	97 (32/33)	NA	1 (1/70)	0 (0/70)
ESD with a hook knife[44]	95 (194/204)		0.5 (1/204)	NA	1.5 (3/204)
Submucosal-endoscopic resection with hypertonic saline-epinephrine solution (S-ERHSE)[45]	NA	79 (36/46)	0 (0/46)	4(2/46)	8 (4/46)
ESD with a mixture of high-molecular-weight hyaluronic acid and Glyceol[35]	100 (26/26)		0 (0/26)	3.8 (1/26)	0 (0/26)
ESD with TT knife[28]	88 (14/16)		NA	NA	0 (0/16)

NA: Not analyzed.

used for dissecting the submucosa. If the target to be dissected cannot be seen directly by any way, a transparent attachment on the tip of the endoscope is useful to stretch the connective tissue and improve the field of viewing in the submucosa. Because the submucosal cushion flattens down as time is passed, it is also important to start dissecting the submucosa immediately from the incising part of the mucosa before further marginal mucosal cutting and to inject the prepared solution into the submucosa repeatedly if a security cushion is necessary to be kept.

Effective control of bleeding during the procedure, especially in the step of submucosal dissection is important for safer, faster, and more reliable ESD. It is more desirable to prevent bleeding than to stop it after its occurrence. For vessels, which are smaller than the tip of the knife, non-contact electrocoagulation of the vessels with a flex-knife is usually enough to prevent or stop bleeding without changing to another device. Bleeding from large vessels also can be prevented or stopped by using a hemostatic forceps.

Management of post ESD ulcer

After total removal of the tumor, all visible vessels located in the post-ESD ulcer base are treated using hemostatic forceps. Finally, 20 mL of sucralfate liquid is sprayed using the outer sheath of a rotational endoscopic clip device to confirm the achievement of correct hemostasis and to coat the post-procedure ulcer base[37].

Management after ESD

After ESD, patients are prohibited from eating and drinking until the next day of ESD. If laboratory findings and chest and abdominal X-ray remain unremarkable, the patients are permitted oral soft foods. Follow-up endoscopy is performed within 1 wk to check up post ESD ulcer healing before the patient is discharged from the ward. Proton pump inhibitor and sucralfate are administered until confirmation of healing of the post ESD ulcers. All patients with ESD also undergo a follow-up endoscopy 2 mo after ESD to confirm the healing and exclude recurrence[38-40]. In case of curative ESD *en-bloc* resection, annual endoscopies are performed to detect new metachronous tumors rather than recurrent tumors, since the local recurrence rate is very low[17]. For tumors with non-curative or non-evaluable resection margins, but fulfilling the criteria of node-negative tumors, endoscopy is performed every 6 mo to detect local recurrent tumors, at least for the first three years of follow-up.

Outcomes of endoscopic submucosal dissection

In comparison with outcomes of conventional EMR (approximately 75% of the *en bloc* resection rate, a high risk of local recurrence ranging from 2% to 35%[41], those of ESD are extremely good. As shown in Table 2, the *en bloc* resection rate is more than 90% regardless of the size while the local recurrence rate is almost zero[8,28,35,42-45].

Complications of endoscopic resection include pain, bleeding, perforation, stricture formation, *etc.* Bleeding is the most common complication, which is typically minor and treatable with endoscopy. The risks vary according to the definition of bleeding (Table 2). Most bleeding occurs during the procedure or within 24 h and predominantly in tumors located in the upper third during the procedure or the lower third of the stomach after the procedure[42]. Our preliminary data show that 13 of 382 resections (3.4%) are complicated by post-ESD bleeding. Eight bleedings (62%) occur from the post-ESD ulcers located in the lower third, 4 from the middle third and 1 from the upper third of the stomach. Eight bleedings (62%) also occur within 24 h after ESD, 2 d after ESD, 3 between 6 d and 10 d after ESD. These findings indicate that post-ESD bleeding should be especially checked for within a day after ESD when the tumors are located in the lower third of the stomach.

Perforation is another major complication related to ESD. As a result of the techniques, the perforation rate of ESD has decreased to 0%-8%[7,22,25,28,35,42,43] (Table 2). Furthermore, recent case series suggest that immediately recognized perforation can be successfully sealed with endoclips as conservatively observed without emergency laparotomy by endoscopic clipping, nasogastric suction, decompression of pneumoperitoneum, and antibiotics [46].

CONCLUSION

Endoscopic resection has become a reasonable and convenient diagnostic and treatment modality, because

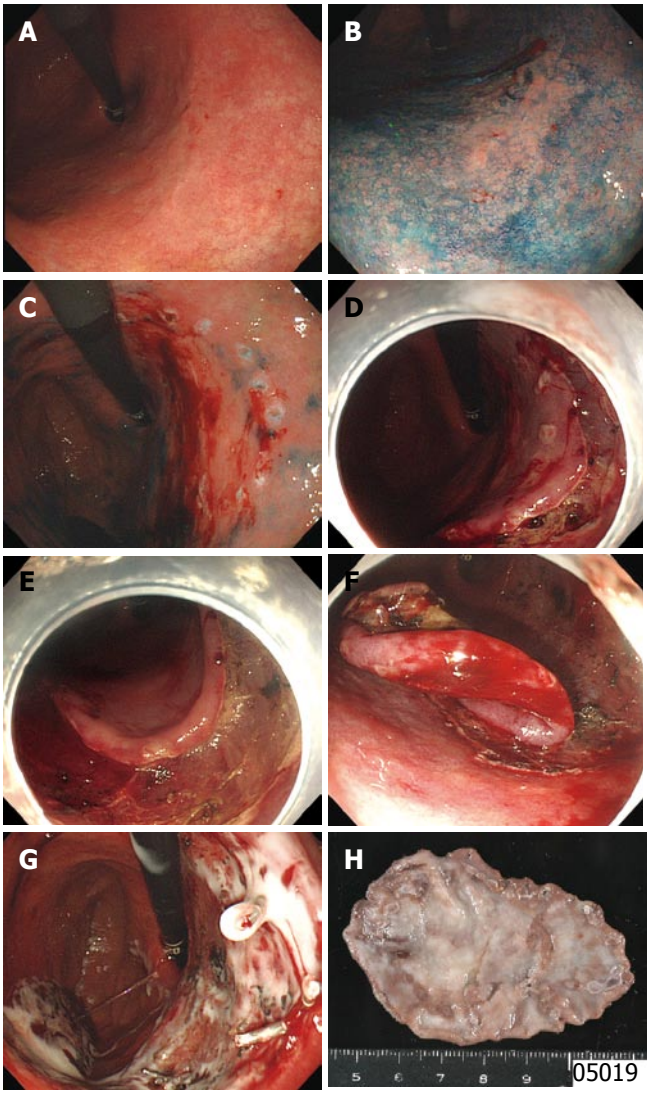

Figure 2 Endoscopic submucosal dissection (ESD). **A**: Ordinal endoscopy showing a whitish slight elevation with a blurred margin in the lesser curvature of the middle gastric body; **B**: Chromoendoscopy revealing margins of the lesion clearly; **C**: Marking dots on the circumference of the lesion; **D**: The incised mucosa around the marking dots of the distal margins; **E**: Before completion of circumferential mucosal incision, submucosal dissection from the distal edges; **F**: After mucosal incision with slight submucosal dissection circumferentially, submucosal dissection from the edge of the posterior wall to the anterior wall; **G**: Complete detachment of the lesion from the muscle layer and spraying sucralfate for confirmation of hemostasis; **H**: The resected specimen including the whole marking dots showing *en bloc* resection of the lesion.

histological information about the whole tumor can be obtained and furthermore, a curative treatment is achieved in case of localized tumors without lymph node metastasis, preserving the whole stomach. From this point of view, ESD enables us to resect not only small tumors, but also large or ulcerative tumors endoscopically. ESD has also brought us the concept of diagnostic endoscopic resection for some tumors clinically diagnosed as submucosal invasive cancers, because histopathological diagnosis of submucosal invasive cancers lacks consistency with clinical diagnosis in 66% of cases[47]. If we can perform thorough and precise histopathological investigations using the resected specimens in an *en bloc* fashion, there is no way to deny the application of endoscopic resection as the first step before gastrectomy, which would consequently avoid unnecessary gastrectomy. However, some complications related to ESD are still a matter of concern. Further refinements of the technique will ultimately help to achieve the goal of eradication of stomach neoplasms.

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EDITORIAL

Cyclooxygenases in hepatocellular carcinoma

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Abstract

Many epidemiological studies demonstrate that treatment with non-steroidal anti-inflammatory drugs (NSAIDs) reduce the incidence and mortality of certain malignancies, especially gastrointestinal cancer. The cyclooxygenase (COX) enzymes are well-known targets of NSAIDs. However, conventional NSAIDs non-selectively inhibit both the constitutive form COX-1, and the inducible form COX-2. Recent evidence indicates that COX-2 is an important molecular target for anticancer therapies. Its expression is undetectable in most normal tissues, and is highly induced by pro-inflammatory cytokines, mitogens, tumor promoters and growth factors. It is now well-established that COX-2 is chronically overexpressed in many premalignant, malignant, and metastastic cancers, including hepatocellular carcinoma (HCC). Overexpression of COX-2 in patients with HCC is generally higher in well-differentiated HCCs compared with less-differentiated HCCs or histologically normal liver, suggesting that COX-2 may be involved in the early stages of hepatocarcinogenesis, and increased expression of COX-2 in noncancerous liver tissue has been significantly associated with shorter disease-free survival in patients with HCC.

In tumors, overexpression of COX-2 leads to an increase in prostaglandin (PG) levels, which affect many mechanisms involved in carcinogenesis, such as angiogenesis, inhibition of apoptosis, stimulation of cell growth as well as the invasiveness and metastatic potential of tumor cells.

The availability of novel agents that selectively inhibit COX-2 (COXIB), has contributed to shedding light on the role of this molecule. Experimental studies on animal models of liver cancer have shown that NSAIDs, including both selective and non-selective COX-2 inhibitors, exert chemopreventive as well as therapeutic effects. However, the key mechanism by which COX-2 inhibitors affect HCC cell growth is as yet not fully understood.

Increasing evidence suggests the involvement of molecular targets other than COX-2 in the anti-proliferative effects of COX-2 selective inhibitors. Therefore, COX-inhibitors may use both COX-2-dependent and COX-2-independent mechanisms to mediate their antitumor properties, although their relative contributions toward the *in vivo* effects remain less clear.

Here we review the features of COX enzymes, the role of the expression of COX isoforms in hepatocarcinogenesis and the mechanisms by which they may contribute to HCC growth, the pharmacological properties of COX-2 selective inhibitors, the antitumor effects of COX inhibitors, and the rationale and feasibility of COX-2 inhibitors for the treatment of HCC.

Key words: Cyclooxygenase-2; Cyclooxygenase-1; Hepatocellular carcinoma; Non-steroidal anti-inflammatory drugs ; Inhibit cyclooxygenase-2

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INTRODUCTION

Hepatocellular carcinoma is one of the most common malignancies worldwide, accounting for approximately 6% of all human cancers and 1 million deaths annually, with an estimated number of new cases of over 500 000 per year[1,2]. Although the clinical diagnosis and management of early-stage hepatocellular carcinoma (HCC) has improved significantly, HCC prognosis is still extremely poor and the cellular mechanisms contributing to hepatic carcinogenesis are relatively unknown. Therefore, investigating HCC pathogenesis and finding new diagnostic and treatment strategies is important.

Various risk factors have been associated with HCC, such as hepatitis B (HBV) and hepatitis C (HCV) viral infections, alcohol consumption and aflatoxin B1 (AFB1) intake. HBV and HCV infections are the most frequent underlying causes of HCC. However, although a number of experimental observations underline the

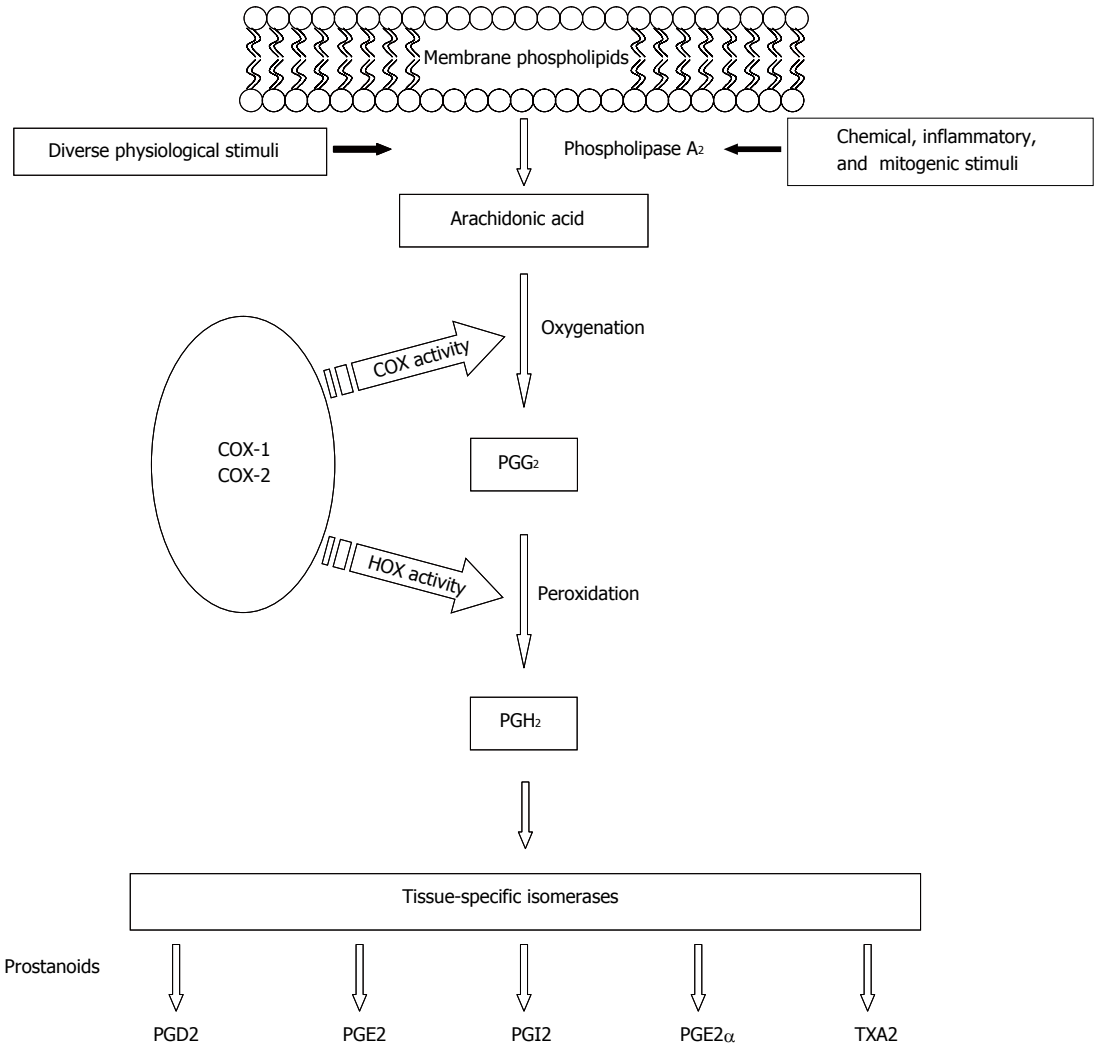


Figure 1 Prostanoids biosynthetic pathway.

potential for viral products in contributing to hepatocyte transformation, only in a minority of patients among the many suffering from chronic viral hepatitis and cirrhosis is there a neoplastic transformation in a given time lapse, suggesting that other co-oncogenic events are probably involved in the multistep process of hepatocyte transformation *in vivo*. HCC development is in fact a complex process associated with an accumulation of genetic and epigenetic changes that pass through the steps of initiation, promotion and progression.

Chronic inflammation is a recognized risk factor for carcinogenesis. Indeed it is thought to play a role in the pathogenesis of several types of cancers, such as cervical cancer, ovarian cancer, oesophageal adenocarcinoma, mesothelioma, colorectal cancer, lung cancer and also HCC[3]. The ability of inflammation alone to cause malignancy is supported by the fact that other non-viral, inflammatory diseases of the liver such as alcoholic hepatitis, hemochromatosis, and primary biliary cirrhosis can also predispose to the development of hepatocellular carcinoma. Therefore, hepatic inflammation, due to viral and also non-viral chronic liver diseases, may represent an early step in the development of malignancy with genetic changes occurring as a later manifestation of a prolonged (chronic) inflammatory process. Inflammatory-mediated events, such as the production of cytokines, reactive oxygen species (ROS), and mediators of the inflammatory pathway, such as cyclooxygenase-2 (COX-2), may therefore contribute to tumor formation. Recent evidence indicates that COX-2 is an important molecular target for anticancer therapies, and COX-2 inhibitors appear to have anticancer effects in different types of malignancies.

FUNCTIONS AND STRUCTURE OF THE CYCLOOXYGENASES

At least two distinct cyclooxygenases are present in humans, COX-1 and COX-2. COX enzymes, also referred to as prostaglandin H synthases, or prostaglandin endoperoxide synthases, are the rate-limiting enzymes that catalyze prostaglandin (PG) and thromboxane (TX) synthesis from 20 carbon polyunsaturated fatty acids, most commonly arachidonic acid (AA), which are released from membrane-bound phospholipids, usually by the action of phospholipase enzyme A_2 (Figure 1). Next, oxygenation of AA by COX produces an unstable intermediate, prostaglandin G_2 (PG_2), which is converted to prostaglandin H_2 (PGH_2) by the peroxidase activity

of COX. PGH_2 is subsequently converted to other PGs (PGD_2, PGE_2, $PGF_2\alpha$, PGI_2) or thromboxanes (TXA_2). The array of PGs produced varies according to the downstream enzymatic machinery present in a particular cell type (Figure 1).

COX enzymes are proteins with a molecular weight of about 68 kilodaltons (kDa) in an unmodified condition, which increases to 72-74 kDa after post-translation glycosilation[4]. The structure of COX enzymes consists of three distinct domains: an N-terminal domain with a conformation that is highly similar to that of epidermal growth factor, a domain containing a series of amphipathic helices, which comprise the membrane attachment site, and a C-terminal catalytic domain, which contains the cyclooxygenase and peroxidase active sites.

Although the two enzymes are highly similar in structure and enzymatic activity they have different genomic structures and different gene regulations and expressions. COX-1 was first purified and characterized in the 1970s and the gene was isolated in 1988[5-7], whereas the COX-2 gene was cloned in 1993[8]. COX-1 and COX-2 are encoded by separate genes located on different human chromosomes. The gene encoding for COX-1 enzyme is located on chromosome 9 (9q32-9q33.3) and is approximately 40 kilobase (kb) pairs, contains 11 exons and its mRNA is 2.8 kb[9]. The gene encoding for COX-2 is located on chromosome 1 (1q25.2-25.3), contains 10 exons and is approximately 8.3 kb with a 4.5 kb transcript[10].

The COX-1 gene exhibits the features of a housekeeping gene, it lacks a TATA box[11], and is generally not subject to transcriptional induction, but it is constitutively expressed with near-constant levels and activity in most tissues and cell types.

COX-2 is an inducible or early-response gene, whose expression is undetectable in most normal tissues. COX-2 is highly induced in response to a broad spectrum of stimuli such as bacterial lipopolysaccharide (LPS)[12], cytokines[13], and growth factors[14,15]. The inducibility of COX-2 can be explained by the presence, in the 5' -flanking region of its gene promoter, of several potential transcription regulatory sequences, including a TATA box and multiple transcription factor binding sites (C/EBP, AP-2, SP1, NF-κB, CRE, Ets-1, PEA-3 and GATA-1)[16,17]. Transcriptional control of the COX-2 gene is cell-specific, and it is evident that more than one pathway may co-operate to regulate COX-2 expression. As reported by Araki[18], in human hepatocellular carcinoma cells, increased COX-2 mRNA and protein expression may result from the combined de-regulation of Wnt and Ras pathways. In addition, in the adult liver, hepatocytes show a behavior pattern unique among cells that respond to inflammatory stresses. In contrast to fetal hepatocytes, which express COX-2 in response to proinflammatory stimuli[19], such as LPS and proinflammatory cytokines, adult hepatocytes fail to express COX-2 regardless of the type of challenge[20]. The presence of high levels of C/EBP-α seems to be involved in the impairment of COX-2 expression in these cells when challenged with proinflammatory stimuli[20]. Therefore, the expression of COX-2 associated with liver diseases, such as cirrhosis and HCC, could be considered a marker of dedifferentiation in adult hepatocytes.

COX-2 gene expression is also subject to negative regulation. Indeed, COX-2 expression can be inhibited by glucocorticoids, IL-4, IL-13 and the anti-inflammatory cytokine IL-10[21-23].

COX-2 expression can also be regulated at post-transcriptional levels in tumors. In the 3' untranslated region (3'-UTR) of the COX-2 mRNA there are multiple copies of the AUUUA motif, which are known to be involved in the control of both mRNA stability and protein translation. Such motifs represent potential targets by which various agents can stabilize or destabilize the COX-2 mRNA, and this may ultimately lead to an increase or decrease in enzyme activity levels. It has been shown that some proteins, such as tristetraprolin[24] and AUF1[25], which also bind to the 3'-UTR, can decrease levels of the COX-2 mRNA. In contrast, other proteins such as HuR, a RNA binding protein prolong the half-life of COX-2 mRNA in colon cancer by binding to the COX-2 AU rich element[26,27]. High levels of HuR protein have also been reported in HCC cell lines and therefore could be responsible for COX-2 overexpression in this tumor[28].

As mentioned before, hepatitis C and hepatitis B virus infections are the major etiological agents of chronic liver diseases, which can lead to the development of liver cirrhosis and HCC. However, it is not well known how HBV and HCV are individually involved in human hepatocarcinogenesis. Recent studies have shown that both viruses are able to promote COX-2 expression. After integration of the HBV DNA into the host genome, the expression of the viral protein HBx upregulates COX-2 expression by transactivation of the COX-2 gene promoter through the NF-AT transcription factor[29,30]. This study therefore demonstrated that COX-2 might be an important cellular effector of HBx protein, which is often the only viral protein expressed by transformed hepatocytes in HCC caused by HBV infection. In addition, the endoplasmic reticulum stress response, due to the expression of the HBV surface protein, may also lead to COX-2 expression through the activation of NF-κB and p38 MAPK[31]. Similarly, a recent study showed that infection with HCV induces the production of ROS and subsequent activation of NF-κB, which in turn mediates COX-2 expression and subsequent PGE_2 production[32]. These studies, therefore, provide new insights into the mechanisms by which hepatitis viral infection, through increasing COX-2 expression and PGs production, might be relevant to the development of liver diseases and hepatocarcinogenesis.

It has been suggested that there is another COX enzyme formed as a splice variant of COX-1[33], referred to as COX-3. COX-3 is made from the COX-1 gene but retains intron 1 in its mRNA. Its expression was initially reported in the canine cerebral cortex and in lesser amounts in other analyzed tissues[33]. Recent molecular biology studies revealed that indeed three distinct COX-1 splicing variants exist in human tissues[34]. The most prevalent of these variants, called COX-1b1, arises via retention of the entire intron 1, leading to a shift in the reading frame and premature termination. This would make the expression of a full-length protein impossible, therefore a catalytically active form of the enzyme might

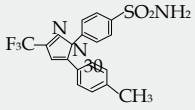
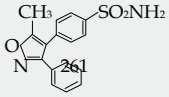
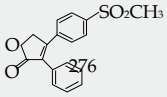
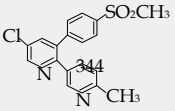
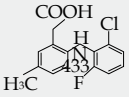
Brand	**Celebrex**	**Bextra**	**Vioxx**	**Arcoxia**	**Prexige**
Generic:	Celecoxib	Valdecoxib	Rofecoxib	Etoricoxib	Lumiracoxib
Chemistry:	Sulphonamide	Sulphonamide	Sulphonyl	Sulphonyl	Phenylacetic acid
					
COX-1/COX-2 ratio	30	261	276	344	433
Pharmacokinetics:					
Oral bioavailability (%)	22-40	83	92-93	100	74
Tmax (h)	2-4	2.3	2-3	1	2-3
Half-life (h)	11	8-11	10-17	22	3-6
Vol.Dist. (L)	455	86	86-91	120	9
Plasma protein binding (%)	97	98	87	92	> 98
Metabolism					
Main pathway	Oxydation CyP450 (2C9, 3A4)	Oxydation CyP450 (2C9, 3A4)	Cytosolic reduction	Oxydation CyP450 (3A4)	Oxydation CyP450 (2C9)
Urinary excretion (%)	29	70	72	60	54

Figure 2 Pharmacological features of coxibs.

not exist in humans. However, the other two variant types, called COX-1b2 and COX-1b3, although retaining the entire intron 1, lack a nucleotide in one of two different positions, thereby encoding predicted full-length and probably COX-active proteins, as suggested by functional studies, which revealed that COX-1b2 is able to catalyse the synthesis of PGF2α from AA[34].

COX INHIBITORS

NSAIDs have long been known as drugs that have the three favorable analgesic, anti-pyretic and anti-inflammatory effects. However, NSAIDs differ in their therapeutic potency, gastrointestinal side effects and COX inhibition ratios. NSAIDs cover a wide range in their ratios of inhibitory potencies (i.e. selectivity) towards COX-1 and COX-2. Some NSAIDs have moderate selectivity for COX-1 (e.g., ketorolac, flurbiprofen, ketoprofen, piroxicam), others inhibit both COX isoforms (dual inhibitors; e.g. indomethacin, aspirin, naproxen, ibuprofen), other NSAIDs favor COX-2 inhibition (e.g. sulindac, nimesulide etodolac, meloxicam), and finally the newest ones are highly selective for COX-2 (COXIB; e.g. celecoxib, rofecoxib, lumiracoxib, valdecoxib, etoricoxib) (Figure 2). Although the mechanism of action of the different COXIB is similar, their chemical structures differ. In addition, the pharmacokinetics and metabolism of each individual COXIB are unique (Figure 2)[35,36].

COX IN HEPATOCELLULAR CARCINOMA

Strong support for a connection between COX-2 expression and carcinogenesis has come from genetic studies. The number and size of intestinal polyps in $APC^{\Delta 716}$ mice, a murine model of human familial adenomatous polyposis coli (FAP), were reduced in animals that were engineered to be also COX-2 deficient[37]. In a separate study, homozygous deficiency of COX-2 reduced skin tumorigenesis in a multistage mouse skin model[38]. On the contrary, overexpression of COX-2 was sufficient to induce tumorigenesis in transgenic mice[39-41].

The evidence that COX-2 may be a logical therapeutic target in HCC comes from studies that showed overexpression of COX-2 in patients with HCC[42-46]. COX-2 expression is generally higher in well-differentiated HCCs compared with less-differentiated HCCs or histologically normal liver, suggesting that COX-2 may be involved in the early stages of hepatocarcinogenesis[42,44,46]. In addition, a significant correlation between COX-2 expression and active inflammation in the adjacent noncancerous liver has been reported[43,47], and increased expression of COX-2 in noncancerous liver tissue was significantly associated with shorter disease-free survival in patients with HCC[43]. This result is of great importance from a clinical point of view, as it suggests that COX-2 expression may play an important role in the relapse of HCC after surgery.

Furthermore, we recently reported that COX-2 expression in the tumor tissue was significantly correlated to the presence of inflammatory cells, macrophages and mast cells[46]. However, COX-2 expressing cells and the number of both types of inflammatory cells decreased with progression of the disease, suggesting their possible involvement in the early stages of hepatocarcinogenesis.

The decrease in COX-2 expression during tumor progression as observed in HCC is unusual. A possible explanation for this different behavior pattern is that, in some cell types, COX-2 overexpression may cause a growth disadvantage, as suggested by Trifan[48], who reported that COX-2 overexpression may induce cell cycle arrest in a variety of cell types.

Although less attention has been drawn to the potential role of the constitutive COX-1 enzyme in carcinogenesis, recent evidence supports its implication in skin and intestinal tumorigenesis[38,49-52]. COX-1 is up-regulated in human breast[53], prostate[54], cervical[55] and ovarian cancers[56,57]. On the other hand, loss of the COX-1 gene results in reduced intestinal tumorigenesis in Min mice[49].

We recently analyzed COX-1 expression in HCC and the surrounding non-tumor tissues[58]. On the whole, we found a higher COX-1 expression in the cirrhotic liver

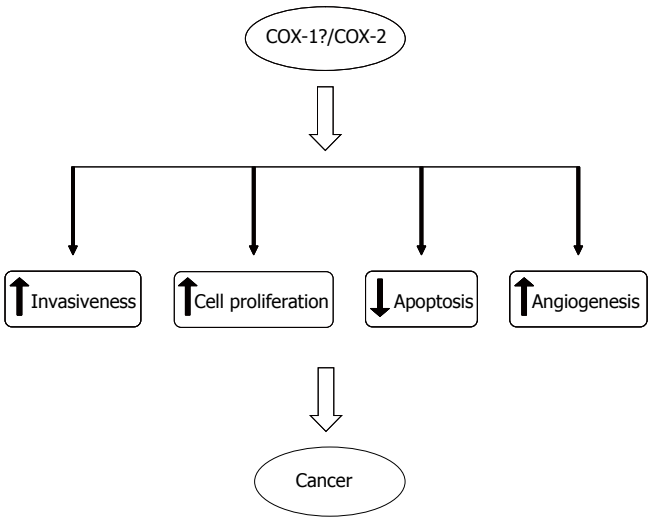


Figure 3 Effects of COX enzymes on different cellular dynamics.

tissues surrounding HCC than in the tumors. However, in some cases COX-1 was up-regulated in the tumor tissues compared to the adjacent non-tumoral cirrhotic tissues. In well-differentiated HCC, COX-1 expression was significantly higher than in the poorly-differentiated tissues, suggesting that the presence of COX-1 might be also involved in the early stages of tumor growth.

COX INHIBITORS IN HEPATOCELLULAR CARCINOMA

Evidence from animal models

Experimental studies on animal models of liver cancer have shown that NSAIDs, including both selective and non-selective COX-2 inhibitors, exert chemopreventive as well as therapeutic effects[59-64]. In the rat model of choline-deficient, L-amino acid-defined diet (CDAA)-induced hepatocarcinogenesis the administration of aspirin or nimesulide with the diet decreased the number of preneoplastic and neoplastic nodules[60,63]. In a recent study by Marquez-Rosado[64] treatment with celecoxib was highly effective in inhibiting the multiplicity and size of liver preneoplastic lesions induced by DEN, 2-AAF and partial hepatectomy.

The therapeutical potential of the specific COX-2 inhibitors, such as celecoxib and meloxicam, in HCC generated in nude mice has also been shown[65,66]. The treatment significantly reduced the growth of HCC *in vivo* by enhancing tumor cell apoptosis and reducing proliferation.

Overall, these results suggest that NSAIDs and other selective COX-2 inhibitors may be of value in the chemopreventive as well as therapeutic activities against liver cancer.

Evidence from "in vitro" experiments

The involvement of COX-2 in carcinogenesis is believed to be primarily mediated through its influence on cell proliferation, apoptosis, angiogenesis and cell invasiveness[67] (Figure 3).

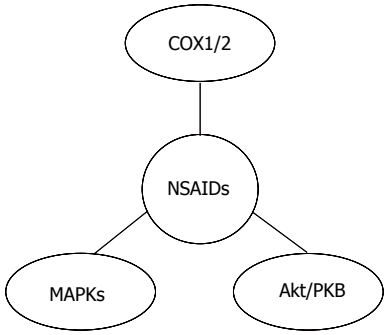


Figure 4 Molecular targets of NSAIDs in HCC.

The role of COX-2 in the stimulation of cell proliferation can be attributed to its involvement in the production of prostaglandins. Indeed, evidence indicates that PGs promote cell proliferation, and conversely the growth-inhibitory effects of COX inhibitors can be reversed by exogenous addition of PGs. It has been demonstrated that prostaglandins increase DNA synthesis and cell proliferation of rat hepatocytes[68,69], and of human HCC cells[45].

On the other hand, it has been demonstrated that COX-2 inhibitors are able to suppress HCC cell growth[44,45,58,70-74]. Several mechanisms have been proposed for the antitumor effects of NSAIDs in HCC. However, the key mechanism by which COX-2 inhibitors affect HCC cell growth remains unclear. Some studies have shown that NSAIDs are able to inhibit HCC cell growth by cell cycle arrest[72,73,75], induction of apoptosis[44,73,74] or necrosis[72].

Recent evidence indicates that pharmacological inhibition of COX-1 activity by selective COX-1 inhibitors also blocks cell growth, promotes apoptosis and inhibits the cell cycle in ovarian[57], breast[76], bladder and prostate[77] cancer cells. In addition, a combination of COX-1 and COX-2 selective inhibitors was found to suppress polyp formation more effectively in the intestinal tumorigenesis of the *Apc* knockout mouse model[52]. Interestingly, we recently showed that the selective COX-1 inhibitor SC-560 inhibits cell growth and induces apoptosis in HCC cells[58]. Moreover, the combination of the COX-1 inhibitor with selective COX-2 inhibitors, resulted in additive effects on cell growth inhibition. These results suggest that both COX-1 and COX-2 inhibitors may have potential therapeutic implications in HCC patients.

However, it is still controversial whether the antitumor effects of COX-2 inhibitors in HCC are due predominantly to the inhibition of COX-2 activity[45,58]. Indeed, the antineoplastic effect of NSAIDs might not be mediated only by COX-2 inhibition, but NSAIDs might act on different molecular targets as well[78].

Increasing evidence suggests the involvement of molecular targets other than COX in the antitumor effects of selective inhibitors also in HCC, including the mitogen-activated protein kinase (MAPK)[79] and the PI3K/Akt pathway[45,70] (Figure 4). The existence of COX-independent mechanisms of NSAIDs action is further supported by the evidence that their antineoplastic effects are observed with concentrations that are greater than

those necessary to fully inhibit the synthesis of PGs, and by the observation that they inhibit HCC cell proliferation in COX-2 negative cells[79]. Interestingly, COX-2-independent effects of celecoxib have also been observed during hepatocarcinogenesis *in vivo*. In the study by Marquez-Rosado[64] neither COX-2 expression nor PGE_2 production were altered by celecoxib treatment, suggesting that celecoxib effects are mediated by COX-2/PGE_2-independent mechanisms.

COX-2 AND HCC ANGIOGENESIS

A substantial body of evidence supports a role for COX-2 in angiogenesis, the "sprouting" of capillaries from pre-existing vasculature, in a variety of human malignancies[80-83]. COX-2 promotes angiogenesis, mainly through the synthesis of prostanoids, which can induce tumor angiogenesis in an autocrine and/or paracrine fashion by stimulating the expression of pro-angiogenic factors[84,85]. However, the precise role of each individual prostanoid remains largely unknown.

COX-2 expression has been reported to correlate with tumor angiogenesis in patients with HCV- or HBV-associated HCC[86,87]. Moreover, in a recent study we showed a positive correlation between COX-2 expression in tumor tissues of HCC patients and the presence of microvessels inside the tumor mass, assessed by staining endothelial cells with anti-CD34 antibody[46]. In addition, we reported that COX-2 was the only independent variable that showed a positive correlation with CD34 in a multivariate analysis, confirming the possible role of COX-2 in HCC angiogenesis. These findings suggest the hypothesis that selective inhibition of COX-2 by treatment with COXIB may contribute to inhibit HCC-associated angiogenesis, and thus provide an additional rational approach for treatment of this malignancy.

COX-2 AND INVASIVENESS OF HCC CELLS

A link between COX-2 expression and invasiveness has been observed in several human malignancies[88,89]. Colon cancer cells that constitutively expressed COX-2 acquired increased metastatic potential that could be reversed by treatment with COX inhibitors[90]. This phenotypic change was associated with increased expression and activation of metalloproteinase-2 (MMP-2)[90]. Similarly, PGE_2 induces MMP-2 expression and activation in HCC cells[91], and treatment with aspirin and with the selective COX-2 inhibitor NS-398 inhibits the HGF-induced invasiveness of HCC cells[92], suggesting the key role of the COX-2/PGE_2 pathway in tumor invasiveness of liver cancer.

COX-2 AND MULTIDRUG RESISTANCE

Growing evidence indicates that COX-2 overexpression can up-regulate the expression of the Multidrug Resistance 1 (MDR1) gene and the levels of its product, the multidrug efflux pump P-glycoprotein (P-gp)[93,94]. COX-2 could therefore contribute to the development of resistance to pharmacological treatment by the tumor cells[93,94]. Recently, the MDR phenotype was associated with COX-2 overexpression in liver cancer cells[95].

It could be speculated that a selective inhibition of COX-2 activity could reinforce the antitumor action of conventional chemotherapy by acting on the expression of P-gp. The rationale behind the possible combination of traditional chemotherapy and selective COX-2 inhibitors is further supported by the fact that chemotherapy itself induces COX-2 expression[96].

CONCLUSION

There is compelling evidence that COX-2, and also COX-1, have a role in hepatocarcinogenesis, but many questions need to be answered. A number of studies have shown that several different mechanisms may account for the anticancer effects of NSAIDs, although the main mechanism remains unclear. The effects of NSAIDs on tumor growth are most likely to be multifactorial, and COX-inhibitors may use both COX-2 and non-COX-2 targets to mediate their anti-HCC activities. Consequently, a better understanding of the COX-2-dependent and COX-2-independent pathways may help to optimize the use of COX-2 inhibitors in the prevention and treatment of HCC.

Recently, concern was raised about the cardiovascular safety of the selective COX-2 inhibitor Rofecoxib[97,98], and as a consequence it was withdrawn from the USA market by Merck and Co. Further investigation is required to define the safety profile of selective COX-2 inhibitors, especially when they are used at high doses and for long periods of time.

An exciting, novel concept in cancer chemoprevention and treatment is the use of a combination therapy. A combination therapy (which may allow dose reduction, and hence decreased systemic bioavailibility) of NSAIDs or COXIBs with agents that specifically modulate relevant biochemical targets of COX-2 inhibitors may take advantage of synergistic growth inhibitory effects against cancer cells and could reduce the toxicity associated with the intake of COX-2 inhibitors. In addition, the use of COX-2 inhibitors, by their action on the MDR phenotype, may enhance the accumulation of chemotherapy agents and decrease the resistance of tumors to chemotherapeutic drugs. Indeed, several clinical trials are under way based on combinations of COXIBs with conventional anticancer treatments (chemotherapy or radiotherapy)[99] and with novel molecular targeting compounds[100].

On the other hand, since experimental studies have provided evidence that PGs are the molecules that mediate the effects of COX overexpression, other molecules involved in PG biosynthesis and signaling might represent potential targets. Recently, pharmacological inhibitors of PGE_2-EP receptors, which have anti-neoplastic activity, have been generated[101]. Therefore, PG receptors and/or PG synthases may represent novel targets for the prevention and treatment of cancer.

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REVIEW

Transfusion transmitted virus: A review on its molecular characteristics and role in medicine

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Abstract

The present review gives an updated overview of transfusion transmitted virus (TTV), a novel agent, in relation to its molecular characteristics, epidemiological features, modes of transmission, tissue tropism, pathogenesis, role in various diseases and its eradication from the body. TTV, a DNA virus, is a single stranded, non-enveloped, 3.8 kb long DNA virus with a small and covalently closed circular genome comprising 3852 bases. It was tentatively designated *Circinoviridae* virus. TTV genome sequence is heterogeneous and reveals the existence of six different genotypes and several subtypes. TTV has been reported to transmit not only *via* parenteral routes, but also *via* alternate routes. This virus has been detected in different non-human primates as well. At present, TTV is detected by polymerase chain reaction (PCR) with no other available diagnostic assays. It shows its presence globally and was detected in high percent populations of healthy persons as well as in various disease groups. Initially it was supposed to have strong association with liver disease; however, there is little evidence to show its liver tropism and contribution in causing liver diseases. It shows high prevalence in hemodialysis patients, pointing towards its significance in renal diseases. In addition, TTV is associated with several infectious and non-infectious diseases. Although its exact pathogenesis is not yet clear, TTV virus possibly resides and multiplies in bone marrow cells and peripheral blood mononuclear cells (PBMCs). Recently, attempts have been made to eradicate this virus with interferon treatment. More information is still needed to extricate various mysteries related to TTV.

Key words: Transfusion transmitted virus; DNA; Virus; Hepatitis; Transfusion

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INTRODUCTION

Transfusion transmitted virus (TTV) is a recently discovered virus which was suspected to be a causative agent of non-A to non-E hepatitis. TTV was first identified in the serum of a patient who was hospitalized with post transfusion hepatitis of unknown etiology in 1997[1]. Initially, TTV was described as a non-enveloped, 3739 bases long and single stranded DNA virus. Based on its genomic characteristics, it was reported to be a parvovirus-like pathogen. Later, studies on the molecular and biophysical characteristics of TTV demonstrated this novel agent as a non-enveloped virus with a small, covalently closed circular genome of single stranded DNA comprising 3852 bases[2-4]. Its buoyant density is significantly different from that of parvoviruses. Also there was a significant sequence difference between TTV and members of the *Circoviridae*[5]. It was proposed that TTV belongs to a new virus family that was tentatively designated *Circinoviridae*[2] para*circoviridae*[6] or the TTV family[7] by different research groups. TTV isolates have an extremely wide range of sequence divergence[8-10] and were tentatively classified into 23 genotypes with sequence divergence of > 30% from one another[11] or into four major phylogenetic groups[7,11]. TTV like mini virus (TLMV) with 2.8-3.0 kb genomic length was also identified in humans and chimpanzees[12-14].

Since the discovery of TTV, studies have been published describing the prevalence of TTV infection in people with acute or chronic hepatitis as well as in blood donors and drug users and also in healthy persons[15-17]. It is apparent that currently it is not possible to ascribe TTV to any specific diseases. TTV can be transmitted parenterally and has been found in plasma and peripheral blood mononuclear cells. However, non-parenteral transmission is also possible as TTV can be excreted in feces[10]. Molecular and phylogenetic analysis of polymerase

chain reaction (PCR) fragments revealed that TTV could be divided into several genotypes that are found worldwide without any direct correlation with geographical distribution of diseases[2, 18-20]. This is an interesting area to investigate different aspects of TTV and several groups are working to extricate many mysteries related to this agent world over. Until now, an abundance of information has been published on TTV in relation to its molecular form and infectious status.

To have a compilation of information and also have a better understanding of TTV, the present article provides a holistic view on various characteristics of this novel agent with particular emphasis on its molecular characteristics, epidemiological features, endemic behavior and pathogenesis as well as prospects of its eradication on the basis of published information.

MOLECULAR BIOLOGY

TTV, a common virus in humans with high prevalence in the general population[21,22] is a single stranded DNA virus. Its the genome was sequenced by Okamoto *et al*[3] almost entirely on the prototype isolate TA278 encompassing 3739 nucleotides and was temporarily believed to be a linear DNA. However, later studies[2] with the GHI isolate and TA 278 isolate[4] have identified a GC-rich missing link of about 100 nucleotides that complete the TTV genome as a closed circular DNA with a length of 3852 nucleotides (nt) and a particle size of 30-50 nm. Thus, TTV is an unenveloped virus whose genome consists of a circular and single stranded DNA molecule of negative polarity and about 3.8 kb length[2]. TTV has an isopycnic density of 1.31-1.34 g/mL in CsCl[2,4]. The TTV genome has two or three possible open reading frames (ORFs) capable of encoding 770 aa (ORF1), 202 aa (ORF2) and 105 aa (ORF3) polypeptides[4]. Analysis of the TTV transcription pattern in COS-1[23] and bone marrow cells[24] has revealed the existence of at least three species of spliced mRNA molecules of 2.9-3, 1.2, and 1.0 kb in length, with common 5' and 3' termini, leading to the creation of new reading frames (ORF3 and ORF4) in addition to the previously described ORF1 and ORF2[25].

Many studies have indicated a high degree of genetic diversity of TTV. The entire genome was sequenced for SANBAN and TA 278 isolates[26]. The genetic organization of the genome was similar in two isolates: two open reading frames (ORF1 and ORF2) were sandwiched by the motifs of TATA box and polyadenylation signal, and a GC-rich short stretch resided at the midst of the untranslated region. No other ORFs longer than 300 nt and common to SANBAN and TA278 were found. The overall nucleotide sequence identity between the two isolates was 56.7%, significantly lower than that (93%) between TA278 and GH1[2]. Interestingly, the nucleotide sequence identity was relatively higher in the untranslated region (73.0%) than in the translated region (52.2%). A great degree of genetic diversity for a group of viruses represented by TTV most likely had a long history of evaluation and adaptation to humans. Despite the extensive sequence divergence of TTV and TLMV in coding regions of the genome, three areas of remarkable conservation have been identified in the part of UTR that contains promoters and splice sites[24]. Sequence conservation is found among all known human isolates of TTV and TLMV as well as those recovered from a range of non-human primates.

Based on its physico-chemical and genomic characteristics[2-4], TTV was proposed as a member of new viral family tentatively named the *Circinoviridae*[3]. Although TTV shares some features, such as a negative-stranded circular DNA genome, with members of the *Circoviridae*[1], the genetic organization of its genome is distinctly different from that of viruses that belong to this family. Therefore, TTV was tentatively classified into the virus family, *Circinoviridae*[3].

GENOTYPES

The TTV genome sequence is extremely heterogeneous. Phylogenetic analysis performed on TTV isolates recovered from several parts of the world revealed the existence of 6 different genotypes[3,27]. The sequence heterogeneity of the TTV genome, however, is more complex. One report described the identification of 16 TTV genotypes[28]. Another study identified 5 additional TTV genetic groups[10]. One of these was found to represent an additional TTV genotype, whereas the other 4 additional genetic groups were significantly distinct from TTV and from one another compared to the original TTV genotypes. This observation suggested that these 4 new genetic groups represent closely related, yet different, TTV-like viral species. The existence of genetic divergence between different TTV isolates beyond genotypes was noted by another group of researchers[8]. It has been hypothesized that a whole "swarm" of numerous TTV-like species circulates in the human population worldwide[29]. Despite this extensive sequence diversity, all variants of TTV share a common genomic organization with three predicted encoded proteins of similar length and likely function.

TTV was originally found in humans; however, recent studies showed that TTV can also be identified in serum specimens obtained from domesticated farm animals[29] and from non-human primates[29, 30]. Phylogenetic analysis using the TTV sequence obtained from animals demonstrated that these sequences belong to already known human TTV genotypes[29,30], although some sequences recovered from nonhuman primates remain unclassified[30]. The results of these experiments demonstrated that chimpanzees may be infected with some TTV-related species that have not been found in humans. An additional phylogenetic analysis using all known TTV sequences, some of which were not classified[26,30] or were classified previously as new TTV genotypes[10], suggested the existence of 16 genotypes or 13 different TTV-like species. The prototype TTV-I[8] sequences were classified into eight genotypes found in humans as well as in non-human primates and farm animals. Moreover, genetically distant variants, namely PMV, SANBAN and SEN viruses have been identified. Frequent homologous recombination, which can occur when a subject is co-infected with two or more isolates, is an important multiplier of the TTV genetic diversity. This

phylogenetic tree contains 13 major groups. Each major group represents sequences that are more distant from the other major group sequences. This observation is strongly supported by the analysis of frequency distribution of evolutionary distances. Comparison of sequences from one major group of branches to all other branches demonstrates that each of the 13 major groups of branches may represent different viral species. All of these viruses are closely related to the prototype TTV strains tentatively designated TTV-I. The other viral species identified with each major branch were also designated TTV with the addition of Roman numerals (e.g. TTV-II, TTV-III) as proposed previously[8].

All 13 major groups of branches or viral species may be arranged into four groups. Group A contains viral species I and IX; group B contains II, III, X, XI, XII, and XIII; group C contains IV and V; and group D contains VI, VII, and VIII. The TT viruses found in humans belong to groups A, B, and C. Group C is composed exclusively of viruses found in humans and group D of viruses found only in non-human primates. The primate TT viruses can be also found in groups A and B. Group A includes two viral species. One of these viruses is the prototype TTV-I, which was originally identified in a Japanese patient with parenterally transmitted hepatitis of unknown etiology[1]. Previously, seven TTV-I genotypes were identified[27,28]. However, based on phylogenetic analysis, TTV-I genotypes 2 and 3 were suggested to be combined as genotype 2/3[8]. This suggestion reduces the number of TTV-I genotypes to 6. Earlier, several new TTV variants were found in non-human primates[29,30]. The phylogenetic analysis performed in this study confirmed that sequences Bo-Ho and Bo-De identified by Vergchoor *et al* belonged to genotype 2/3. However, sequences Ch-Pe and Ch-Br2 constituted two new TTV-1 genotypes[29] whose sequences constituted a new major branch in the phylogenetic tree representing TTV-IX, which is different from, but closely related to the prototype TTV-I. TTV-IX detected in chimpanzees is most closely related to the TTV-I genotype 8 found only in non-human primates[30]. Recently, several new TTV variants were identified in serum specimens from healthy Japanese individuals[31]. Group B contains TTV-II, TTV-III, TTV-X, TTV-XI, TTV-XII and TTV-XIII, which can be found in non-human primates as well as in human specimens. TTV-II, TTV-III, TTV-X and TTV-XIII were found only in humans. Group C is composed of two human viruses, TTV-IV and TTV-V. Group D consists of three new chimpanzee viruses, namely TTV-VI, TTV-VII and TTV-VIII.

HUMAN TRANSMISSION

TTV was first characterized as a blood-borne virus and was thus referred to as a transfusion-transmitted virus (TTV)[3,32]. However, later studies suggested the existence of other routes of transmission also. In fact the mechanism of TTV transmission has not yet been elucidated. The higher prevalence of TTV in persons treated with blood[3] or blood products[32] has suggested parenteral transmission as a frequent route of TTV infection. TTV is common in patients who have an increased risk of infection with blood-borne viruses, such as hemophiliacs (68%), patients on maintenance hemodialysis (46%), and abusers of intravenous drugs (40%)[3]. There is a high prevalence of TTV in blood products. TTV contamination was found in 10 of 18 batches (56%) of factor VIII and IX concentrates manufactured from non-remunerated donors, and in 7 (44%) of 16 batches of commercially available products[32]. These observations suggest that TTV is transmitted by blood and blood products. However, infrequent detection of TTV-DNA in serum samples from prostitutes and homosexual men[17] and the findings of fecal[33] and bile[34] excretion of TTV indicate that TTV may have characteristics different from other blood-borne viruses. The prevalence of TTV in blood donors in different countries varies between 1.9% to 62%[3,22,27,32,35]: 1.9% in England[32], 9.1%-12.8% in United States[27], 12% in Japan[3] , 36% in Thailand[35] and 62% in Brazil[22]. Moreover, the majority of TTV infected people had no history of blood and/or blood products transfusion. The relatively high prevalence of TTV in blood donors and the large proportion of TTV infected patients with no history of transfusion of blood and blood products also suggest that alternative routes of transmission of TTV infection may exist[36]. TTV DNA has also been detected in saliva[36], throat swabs[37], breast milk[38], semen[39] and vaginal fluid[40] thus, supporting routes of transmission other than blood and blood products[33,37].

Excretion of TTV in feces of infected individuals suggests of possible fecal-oral transmission[33]. Some studies have reported placental transmission of TTV[41-43], whereas others have not detected TTV in cord blood and amniotic fluid[44-45]. These studies show the absence of transuterine transmission of TTV. Since children of TTV-infected mothers apparently tend to get infected more often and earlier after birth than children of TTV negative mothers, the role of postnatal transmission of TTV is being considered. Postnatal route of transmission from mother to child and infection *via* frequent social contacts seem to be very important modes of transmission in children[46-48]. Furthermore, variation in the TTV prevalence in children from 5.1% in Japan[49] to 54% in the Democratic Republic of Congo[50] is also suggestive of the possible involvement of some specific environmental factors in the acquisition of TTV infection. The sexual mode of transmission is likely of low effectiveness[51].

ANIMAL TRANSMISSION

Infection with both TTV and TLMV has been detected in various non-human primates[2,52,53]. At the same time, there are reports showing cross-species transmission of TTV genotypes. Human TTV variants can infect chimpanzees and macaques[13,54]. Beyond primates, host range of TTV and TLMV is uncertain. One study has demonstrated frequent TTV infection of domestic animals such as cows, pigs, sheep and chickens[47]. However, it is not known how these species acquire TTV infection. Recently, highly divergent TTV like viruses were detected in pigs, cats and dogs, distinct from those found using the N22 primers[55] suggesting that this virus family may indeed be widely distributed in the mammalian order. There are reports showing high prevalence of TTV infection in captured

chimpanzees and crab eating macaques[56]. These findings suggest that TTV is widespread among wild Chimpanzees living in West Africa. However, this TTV infection was found non pathogenic. Based on analysis of full-length sequence data, this TTV may represent a new TTV-like viral species or genus, although it is closely related to human TTV[56].

TARGET ORGANS

Regarding target organs for TTV infection, TTV-DNA has been detected by both PCR and in situ hybridization in liver and peripheral blood mononuclear cells (PBMC)[57,58]. However, these studies have shown that TTV replicates in liver, but not in PBMC. Simultaneously, TTV was detected and found with replicative intermediates in bone marrow cells from TTV infected patients[24]. This finding and few other studies[59] indicate that TTV-DNA in PBMC corresponds to viral particles passively attached to cell membrane and TTV infects hematopoietic cells but only replicate when these cells are activated. Since the percentage of circulating activated cells is very low, this may be the cause for the lack of detection of TTV replicative intermediates in freshly isolated PBMC from TTV infected patients.

DIAGNOSTIC ASSAYS

The development of sensitive and reliable polymerase chain reaction (PCR) protocols allowed the detection of TTV DNA at a very high prevalence in sera of healthy populations around the world[21,22,29]. Currently, the heteroduplex mobility assay to detect multiple infections with isolates of TTV belonging to different genotypes or subtypes has also been developed. In the simplest application of heteroduplex mobility assay, heteroduplexes are formed by denaturing and reannealing mixtures of PCR amplified DNA fragments from divergent isolates of the same virus. When these products are separated on polyacrylamide gels a homoduplex band plus two slow moving heteroduplex bands are observed. The mobility of heteroduplexes is related to the genetic distance between two strands. This technique was applied earlier to HIV isolates, measles virus, CMV and hepatitis C virus. While detecting TTV DNA by PCR, it was found that repeated freezing and thawing of serum did not have much effect on stability of TTV DNA. Specimens are not required to be aliquoted for repeated testing and retrospective studies[60].

Both TTV and TLMV have sequence heterogeneity. Some TTV subtypes have less than 50% sequence identity. However, there are certain conserved regions. Primers were designed in such a way that most of the subtypes could be detected[61]. Recently, real time PCR based methods with either SYBR Green or TaqMan Probe, designed to quantitate selectively TTV and TLMV, have also been used[61].

EPIDEMIOLOGY

Epidemiological studies have shown that TTV is described worldwide in various populations. The prevalence of TTV viremia in healthy adults of developed countries is in the range of 1%-34%. Prevalence reported from third world countries was found to be higher, typically 40%-70%. In people who have received multiple blood transfusions the virus is almost universally present with more than one subtype in each individual. Table 1 demonstrates the countrywide prevalence of TTV infection in different categories of populations including both healthy persons and patients with various types of diseases[62-75].

TTV INFECTION IN LIVER DISEASES

From preliminary reports two characteristics of TTV infection have emerged rendering it as a potential cause of liver disease. First, Okamoto *et al*[3] demonstrated that TTV-DNA levels in liver tissue were equal to or 10-100 times higher than those in serum, suggesting that this virus replicated in the liver. Second, Nishizawa *et al*[1] reported the appearance of TTV-DNA in the sera of patients with post transfusion hepatitis of unknown etiology to display close correlation with ALT levels. Neither one of these characteristics, hepatotropism or correlation of viral titres with serum ALT, had previously been demonstrated for HGV. However, most subsequent investigations could not confirm their significance in the development of fulminant hepatitis, cryptogenic chronic liver disease and HCC. In addition, the implications of coinfection with TTV in the natural history of chronic HBV or HCV infection are also far from clear. Although TTV can be transmitted by parenteral route, its role in causing posttransfusion hepatitis has not been established[76-79]. The majority of individuals who become TTV-DNA-positive after blood transfusion usually have normal ALT and do not develop chronic hepatitis, although TTV viremia frequently persists for several years. Patients who develop chronic hepatitis are invariably coinfected with HBV or HCV and chronic hepatitis is closely correlated with HBV or HCV infection. This raises the possibility that TTV is merely an innocent bystander rather than a primary hepatitis virus.

In one of the studies[79], the rate of TTV infections was found to be significantly higher among transfused than among non-transfused patients (26.4% and 4.7%, respectively) and the risk of infection increased with the number of units transfused. The rate of TTV infections with non A-E hepatitis (23.2%) was almost identical to the rate among patients who had been transfused, but did not develop hepatitis (21.8%). Of those patients with acute hepatitis C, 40.0% were simultaneously infected with TTV and TTV did not worsen either biochemical severity or persistence of hepatitis C. In non-A-E cases, the mean ALT was comparable among those positive for TTV and those negative. Neither was there a consistent relationship between ALT and TTV-DNA level among these patients[80].

The role of TTV in acute hepatitis is another unresolved issue. In two Japanese studies[81,82] TTV-DNA was identified in 13.6%-43% of cases of non-A-E community-acquired acute hepatitis. However, these positive rates of TTV do not differ statistically from either those obtained among patients with other types of viral

Table 1 Global prevalence of TTV infection in normal subjects and patient populations

S.No.	Country	Group	No. tested	No. Positive (%)	Reference No.
1	Italy	Patients with different clinical diagnosis			62
		Unselected pathologies	221	110 (50)	
		Hemophillia A	33	24 (73)	
		Hemodialysis	36	19 (53)	
		HCV positive patients			
		Normal ALT	30	17 (57)	
		Abnormal ALT	50	24 (48)	
		Cirrhosis	30	9 (30)	
		HCC	13	8 (62)	
		HCV negative patients			
		Non-A non G hepatitis	23	11 (48)	
		Autoimmune hepatitis	11	4 (36)	
		Primary liver diseases	17	11 (65)	
		Cryptogenic extrahepatic diseases			
		Systemic lupus erythematosus (SLE)	34	19 (56)	
		Psoriasis	102	56 (55)	
		Rheumatoid arthritis	60	17 (28)	
2	Italy	Healthy blood donors	100	22 (22)	63
		Hemophiliacs	178	123 (69)	
3	Italy	Patients			64
		HIV I infected mothers	83	29 (34.9)	
		- Intravenous drug users	46	21 (45.6)	
		- Non-intravenous drug users	37	8 (21.6)	
		Uninfected			
		- Infants born to TTV infected mothers	29	8 (27.5)	
4	Italy	HIV Negative			65
		- Blood donors	104	91 (87.5)	
		- Chronic hepatitis C	106	99 (93.4)	
		- Hemodialysis patients	100	100 (100)	
		- Thalassemic patients	36	36 (100)	
		- IVDUs	37	31 (83.8)	
		HIV Positive			
		- IVDUs	102	102 (100)	
		- Homosexuals	58	52 (89.7)	
		- Heterosexuals	50	44 (88.0)	
5	Italy	Haemophiliacs	217	204 (94)	66
6	China	Healthy persons	136	29 (21.3)	67
		Prostitutes	140	46 (32.9)	
7	China	Intravenous drug users	50	14 (28)	68
		- Hemophilics	50	35 (70)	
		- Thalassemics	40	27 (67.5)	
		- Hemodialysis patients	50	13 (26)	
		Household contacts			
		- Spouse	40	3 (7.5)	
		- Non spouse	57	7 (12.3)	
		Acute hepatitis A	52	4 (7.7)	
		Non A-E hepatitis			
		- Acute	12	5 (41.6)	
		- Chronic	9	2 (22.2)	
		- Fulminant	11	5 (45.4)	
		Hepatitis B carriers	200	30 (15)	
		Hepatitis C carriers	100	36 (36)	
		Healthy adults	100	10 (10)	
8	China				69
		Healthy children	122	33 (2.7)	
		Non A-E hepatitis	19	8 (42.1)	
		- Acute	13	6 (46.1)	
		- Chronic	3	1 (33.3)	
		- Fulminant	3	1 (33.3)	
		Thalassemic children	64	47 (73.4)	

S.No.	Country	Group	No. tested	No. Positive (%)	Reference No.
		- Transfused during cardiac surgery	80	37 (46.3)	
		- Chronic HBV carrier	30	10 (33.3)	
		- Biliary atresia	32	5 (15.6)	
9	USA (Minnesota)	Healthy donors with elevated ALT	99	5 (5)	70
		Healthy donors with normal ALT	146	1 (0.7)	
10	Japan	Patients with chronic liver disease of unknown etiology	69	57 (83)	71
		Volunteer blood donors	50	40 (80)	
11	Tanzania	Rural women	156	115 (74)	72
12	India	Sewage water	63	8 (12.7)	73
13	Brazil	Patients sera	184	48 (26)	74
		Patients saliva	167	49 (46)	
14	Norway	Blood donors	201	180 (98.6)	75

HCC: Hepatocellular carcinoma; IVDUs: Intravenous drug use.

hepatitis or among healthy volunteers. In addition, the ALT levels do not show any difference between TTV-positive and TTV-negative patients. Furthermore, the presence of TTV infection had no apparent effect on the clinical course of patients with hepatitis A, B or C. Thus, according to these studies no correlation appears to exist between TTV infection and the clinical features of sporadic hepatitis. Contrasting another Japanese study[83], TTV-DNA was detected in 2 out of 7 (29%) patients with acute hepatitis of unknown etiology, but in none of the 4 patients with acute HCV-associated hepatitis. At least half of all cases of fulminant hepatitis are seronegative for hepatitis A-E viruses. TTV has been fond in 27%-50% of patients with fulminant hepatitis[3,16,84], but such patients probably received multiple transfusion before testing and recent TTV infection has not always been established. Therefore, it is unclear whether TTV infection is secondary to transfusion or plays an etiologic role in fulminant hepatitis.

Current data suggest that TTV is not the causative agent of chronic liver disease of unknown etiology and neither does it affect the degree of liver damage when present as a coinfection with HBV or HCV[16,77,84-86]. According to our previous study[86], for example, TTV-DNA was detected in 20% of the HBV-positive and 19.5% of the HCV-positive chronic liver disease patients, in 8.3% of seronegative chronic liver disease patients, in 8.3% of seronegative chronic hepatitis/cirrhosis patients and 7% blood donors. Yet, no significant differences between TTV infected and non-infected patients were found as to demographic data, assumed source of infection, biochemical abnormalities, or severity of liver histology. Thus, regarding etiology and progression towards serious chronic liver disease, its contribution seems to be minor if not altogether non-existent. Concerning antiviral therapy, there are no data or treatment of patients who are infected with TTV alone since the role of TTV as a cause of chronic hepatitis has yet to be determined. Studies of patients infected with both HCV and TTV who were treated with interferon showed that the responsiveness to therapy was correlated with HCV alone. In addition, certain genotypes of TTV were quite resistant to interferon although interferon effectively reduced HCV in the same patients[87].

Because the prevalence of TTV is high among patients with chronic viral hepatitis and cryptogenic liver disease, a similar situation has been anticipated to persist among patients with HCC. However, the prevalence of TTV in Thai patients with HCC has shown a wide range of divergence, for example, 6%-60% and 5%-50% in cases with HBV and HCV markers respectively, and 1%-67% in cases without HBV and HCV markers[27,88]. Our study demonstrated the majority of TTV infected HCC to harbor double or triple infections with HBV and/or HCV and the prevalence of TTV infection was comparable to that of healthy volunteers[89]. Contrasting that, another group reported TTV-DNA to occur more frequently in patients with liver cirrhosis and HCC than in those with chronic hepatitis[27]. Using a case-control study to compare the prevalence of 174 Italian patients and matched controls, it was demonstrated that individuals infected with TTV did not exhibit an increased relative risk for developing HCC[90]. Furthermore, it has been demonstrated that the TTV genome is not found integrated into host hepatocyte DNA[91], the one process that might represent a potential risk factor in the development of HCC.

TTV INFECTION IN RENAL DISEASES

Using the polymerase chain reaction (PCR), epidemiological studies have indicated a worldwide distribution of this virus, with prevalence surveys in the general population reporting values of 12% to 19% in Japan[3,92], 36% in Thailand[27], 2% to 10% in European countries[32,85] and 1% in the USA[16]. In patients on maintenance hemodialysis (HD), who are at an increased risk of parenterally transmitted hepatitis virus infection, a high prevalence (32%-53%) of TTV infection has been reported[93,94]. However, the transmission route of the virus is still unknown and the question of any association between duration of HD or previous transfusion and TTV infection is still a matter of controversy[77,95]. There is also little information about the occupational risk of TTV infection in HD unit workers. By using logistic regression analysis, it was shown that a prior blood transfusion and time on HD were not predictors of the presence of TTV-

DNA, so that TTV may have a transmission route not shared by HBV, HCV or HGV. The possibility of TTV transmission, *via* a nosocomial route in HD units, must be considered[77]. One of the possible routes of transmission in an HD unit is direct from person to person. For this reason, the healthcare staff in the HD unit was suspected as being at high risk for TTV infection. Although a low risk of TTV infection was suggested in hospital staff, there is little information on HD unit workers[96]. TTV infection rate was not influenced by age, sex, or mean duration of dialysis[77,79,97]. Nosocomial transmission may account for TTV infection in some patients on hemodialysis[98].

TTV-DNA genotype 1 (G1) was found to be the main TTV DNA genotype in hemodialysis patients. The fact that hemodialysis patients are polytransfused makes it likely that they are at risk of multiple exposures. Therefore, it was interesting that a significant number of patients were apparently coinfected with different strains of TTV. Sequence analysis of clones from two patients with apparent mixed infections showed that TTV strains belonging to two different major genotypes could coexist in a single patient. It was suggested that infection with one TTV type does not protect against infection with another TTV type[99]. The preliminary data suggested that TTV is transmitted mainly *via* a parenteral route[3,85]. When TTV infection was studied in hemodialysis patients who were monitored for HCV infection, co-infection was found in 48% at enrollment. The follow-up of the renal transplant patients revealed, that the persistence of single TTV variants over a long period after organ transplant was common. Considering the heterogeneity of TTV isolates, this finding is against frequent infection with different nucleotide sequences and so horizontal spread of certain variants could have been expected. Nevertheless, in the examined patient group, permanent infections, with only single nucleotide changes in the consecutive samples of the same patient could be observed and the TTV variant detected in one patient was usually remotely related to the TTV variants infecting the others with 58%-97% nucleotide sequence identity between the variants. As a highly variable region of the TTV genome, the N22 region is a candidate to carry humoral epitopes on the surface of the virions. Mutations in this region were, however, infrequently detected. The host's immunity is a plausible evolutionary driving force of the development of TTV genotypes and variants, which process affects most probably the hypervariable genomic regions. If so, the iatrogenic immunosuppression of the transplant recipients can contribute to the long persistence of one variant, while novel infections are infrequent[100].

TTV COINFECTION

Accumulating molecular and clinical evidence indicated that the effects of HIV infection can be modified by coinfection with other viruses[101-104]. However, limited information is available about the prevalence and possible pathogenic role of TTV in HIV infected patients with or without AIDS in relation to specific risk factors, and about the viral titres of TTV. Puig-Basagoiti *et al*[105] reported no influence of TTV infection on CD4 T cell counts and clinical or immune status in HIV-infected patients. In a recent study by Martinez *et al*[106], no relationship was found between TTV DNA detection and HIV category, CD4 count, HBV and HCV infection or demography features. By contrast, Christensen *et al*[101] reported correlation between a low CD4 T cell count and high TTV titre in Danish patients with HIV infection as well as a possible prognostic significance of TTV viral load in immunocompromised patients. High TTV viremia levels were found to be associated with decreased survival rates. A significantly higher rate of TTV positivity was noted in HIV-infected patients than in HIV-negative healthy individuals by two distinct PCR methods. Although the detectability of TTV by either of the two PCR features was as in previous studies[105,106], the relative rate of TTV DNA in the patients studied was found to be associated with the HIV viral load and CD4 cell level as well as the development of AIDS.

A series of recent reports has indicated that viral infections can influence the pattern of autoantibody expression in patients with autoimmune diseases. Neidhart and coworkers[107] demonstrated differences in the autoantibody pattern of patients with SSc with antibodies to cytomegalovirus. Other investigations showed a significantly lower prevalence of rheumatoid factors in RA patients infected with TTV, in comparison with non-infected patients. Hajeer *et al*[108] reported a negative correlation between anti-parvovirus B19 antibodies and rheumatoid factors in patients with RA. In several studies, the autoantibody pattern found in patients with SSc was found to be similar[109-111]. Comparison of the autoantibody patterns in virus infected and non-infected patients with SSc showed that continuing GBV-C or TTV infection or both, have no evident effect on the manifestation of autoantibodies. In conclusion, various reports showed neither a higher prevalence of GBV-C RNA and/or TTV DNA, nor changes in the pattern of expression of autoantibodies in patients with SSc. Therefore, these data provided no evidence for an association between GBV-C and/or TTV infections and SSc.

Not only the clinical significance and the pathogenesis of TTV infection but also the association between TTV infection and raised ALT values have been controversial[1,3,113]. It was found that the raised ALT values were independently related to TTV viraemia among Taiwanese who were not infected with HBV and HCV. TTV infection seemed to be responsible for raised ALT values and to hint positive hepatopathic effects. Tuveri *et al* suggested that TTV might be implicated in a few cases of acute and chronic non A-non G hepatitis[113]. However, Nakano *et al* reported that TTV was not the main causative agent of cryptogenic liver disease[114]. Further efforts at confirming the pathogenecity of hepatocyte damage by TTV are necessary. As for direct correlations between TTV and *H pylori* infection, TTV DNA was detected at a similar rate in patients with and without *H pylori* infection, and *H pylori* infection was detected at a similar rate in patients with and without TTV infection. Similarities of prevalence of TTV between patients with and without infection by *H pylori,* and prevalence of *H pylori* between patients with and without infection by TTV, as well as the discrepancy in

age distributions between prevalence of TTV and *H pylori* in our patients with peptic ulcer disease indicate that no correlation exists between TTV and *H pylori* infection, even though the two agents have similar age distributions in the general population and similar routes of transmission have been suggested for the two agents[115]. According to one report, the high prevalence of genogroup 1 TT virus infection in patients with laryngeal cancer and its striking co-prevalence with human papilloma virus infection is biologically important in the progression of squamous cell carcinoma of the larynx[116].

TTV might replicate in the respiratory tract[117]. Also, although we found no evidence that TTV might be the direct cause of ARD, TTV loads in both nasal swabs and plasma samples were substantially higher in subjects with bronchopneumonia (BP) than in the subjects with milder ARD (laryngitis, bronchitis, and bronchiolitis), suggesting among other possibilities that TTV could be locally or systemically immunosuppressive and aggravate disease induced by other agents[117]. However, there is no information on this matter except for recent report showing an inverse relationship between TTV burdens and CD4 cell counts in patients with human immunodeficiency virus type[101,118,119].

PATHOGENESIS

Although liver tropism has been suggested, TTV also has been found in other organs including kidneys, prostate, mammary glands, brain, bone marrow cells (BMCs) and peripheral blood mononuclear cells (PBMCs)[3,59,120,121]. Although it is not known precisely in which cell(s) TTV replicates, TTV DNA has been detected frequently in the PBMCs[58,120] and it has also been suggested to infect and replicate in hematopoietic cells in the bone marrow[122,123]. Earlier reports had revealed that there was a higher TTV genome load in the PBMCs of cancer patients than in healthy controls (blood donors)[124]. This could have been related to immune abnormality in cancer patients when compared with the controls, thereby allowing increased TTV replication in the former.

It was demonstrated that TTV is present in the nucleus and cytoplasm of some of the PBMCs. It is possible that infection of immune cells could facilitate escape of the virus from the immune response. Concealed as a "Trojan horse", TTV in PBMCs might serve as a reservoir of TTV for chronicity of the infection and transmission in some clinical and epidemiological settings. The observation that TTV-negative PBMCs bound considerable virus *in vitro* suggests that at least some TTV found associated with *ex vivo* derived PBMCs might be of plasma origin rather than produced by the cells themselves. We have, however, obtained evidence indicating that PHA-stimulated PBMCs support TTV replication *in vitro* and release substantial titres of virus into the culture fluid. TTV-related single-stranded DNA viruses such as circoviruses and parvoviruses require actively multiplying cells for productive replication[125,126]. That proliferating hematopoietic cells might be an important source for the TTV that circulates in infected individuals is suggested by findings showing that baseline TTV viremia decreased markedly in virus-positive prospective bone marrow transplant recipients after myelosuppression with cyclophosphamide and total body irradiation[122]. Whether TTV is also dependent on cell cycling for active replication remains to be formally established.

It is well known that some viruses can be activated by immune stimulation, most notably HIV, which requires lymphocyte activation for optimum replication[127,128]. In this regard, it has been shown recently that an animal circovirus related to TTV, porcine circovirus type 2 (PCV-2), can replicate to higher levels in piglets as a result of immunization with a bacterial porcine vaccine[129]. One major distinction between PCV-2 stimulated in this study and TTV is that the porcine virus is capable of causing a wasting syndrome and may do so more effectively following immune stimulation. In addition, immune suppression due to drugs or induced stress resulted in a transient increase in TTV titers in humans[130,131]. This indicated that resting PBMCs could not produce TTV, but that mitogen activated cells could be stimulated in vitro to replicate TTV. Thus, there is a suggestion that TTV levels may be related to the state of activation of the host immune system as postulated in an earlier publication[132].

Recently, replicative circular double-stranded intermediates of TTV DNA have been reported in liver and BMC[24,133]. TTV DNA has been detected by in situ hybridization in nuclei of hepatocytes from experimentally infected rhesus monkeys, indicating that TTV truly infects hepatocytes[134]. However, TTV DNA titers in sera of TTV-infected patients decreased to undetectable levels during immunosuppression following bone marrow transplantation[122]. Moreover, liver cells have been found to contain only TTV DNA and not mRNA[123], suggesting that TTV replicates in hematopoietic cells rather than liver. Southern blot analysis argues against integration of the TTV genome into the genomes of human hematopoietic tumor cells, obtained from bone marrow aspirates, lymph nodes, or human hepatocellular carcinoma[91,135]. Although, TTV DNA is frequently detected in PBMC of infected individuals[58,120], double-stranded replicative intermediates have not been detected in PBMC.

TTV, a new member of the *Circoviridae* family, has not been cultured *in vitro* and its pathogenic potential is still not clear. A cell line for isolating and cultivating TTV will significantly accelerate the research on TTV. Human lymphoblastoid cells, particularly B cells, transformed with oncovirus such as Epstein-Barr virus, may be useful for culturing TTV. Because DNA viruses are often found integrated into host genomic DNA as has been reported for hepatitis B virus[136-139], the possible integration of TTV DNA into the genome of hepatic cells was investigated. When a 2.2 kb TTV probe was used in a Southern blot analysis of liver genomic DNA, no signal was obtained. Thus, TTV DNA was not found to be integrated into hepatocyte chromosomes, and the liver apparently was not the site of TTV replication for this particular case. Similarly, Yamamoto *et al*[91] have reported the absence of viral replication in hepatocytes of TTV infected cases of hepatocellular carcinoma. These data, taken together, suggest that the site of TTV replication occurs in the bone marrow rather than in the hepatocytes, and that TTV

infection was the cause of the aplastic anemia. Similar findings suggesting TTV replication in bone marrow have been obtained by other researchers[88].

ANTI VIRAL THERAPY

After IFN-alpha administration with a regimen of 6 MU thrice a week for 24 wk followed by 3 MU thrice a week for 12 wk, 24 of 50 (48%) concurrent TTV-infected patients achieved complete clearance of TTV DNA 6 mo after the cessation of therapy, with 7%-8% TTV spontaneous clearance rate reported annually in previous reports[77]. Other studies indicated that IFN-alpha has a potential antiviral effect on TTV. In previous studies, IFN therapy was effective against TTV with an eradication rate of 45%-55%[140,141]. Transient disappearance of TTV viremia during IFN-alpha therapy was observed in some reports, demonstrating the direct antiviral effects of IFN-alpha on the suppression of TTV. Nevertheless, delayed TTV clearance (TTV DNA positive at E/T and negative after cessation of therapy) that had not been reported previously occurred in few patients. Delayed complete virological response was observed in chronic hepatitis B patients after the end of therapy with Thymosin alpha 1[142] or IFN[143] that revealed immune modulation effects. Since TTV is a DNA virus as HBV, delayed clearance of TTV after IFN therapy may indicate that immune modulation plays an important role. The findings in the present study implied that both antiviral effects and immunomodulatory actions of IFN-alpha are important on the eradication of TTV. Further studies are needed to investigate and clarify the actual mechanism of responsiveness of TTV to IFN-alpha.

In evaluating the clinical characteristics and virological features related to clearance of TTV after IFN-alpha therapy, the viral clearance at the E/T was the only important factor associated with clearance of TTV viremia. Neither the pretreatment ALT levels nor the histopathology were predictors for TTV clearance. Besides, there was no correlation between response of HCV and TTV. All the results may indicate the difference in virologic kinetics and mechanism of IFN effects between TTV and HCV that influence the response and resistance to IFN-alpha. The ALT levels at E/T or 6 mo after cessation of treatment were not related to TTV but HCV viremia also denied the hepatopathic effects of TTV infection.

OUTLOOK ON TTV

From the compilation of published reports on this newly characterized virus and its global status, it is evident that TTV is prevalent in several countries of the world. As such, it is not involved in causation of a serious problem in the body and simply acts as a bystander without much impact of its single or co-infection with other viruses. Of course, attempts are still going on to find out exact clinical implications of TTV infection. Much is already known about the molecular biology of the virus, yet there still remains a need to develop simple techniques based on molecular and immunodiagnostics to diagnose TTV infection in all categories of laboratories. This will facilitate studies on TTV in more detail and at several places to unravel mysteries related to this infection.

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Increased expression of angiogenin in gastric carcinoma in correlation with tumor angiogenesis and proliferation

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Abstract

AIM: To investigate the implication of angiogenin (ANG) in the neovascularizaton and growth of human gastric carcinoma (HGC).

METHODS: ANG mRNA expression in HGC specimens obtained by surgical resection from patients with HGC were examined by RT-PCR. ANG, Ki-67, VEGF protein expression and microvessel density (MVD) in HGC specimens were detected by immunohistochemistry.

RESULTS: RT-PCR showed significantly higher ANG mRNA expression (0.482 ± 0.094) in HGC tissues than in the surrounding nontumorous tissues (0.276 ± 0.019, $P = 0.03$). MVD within tumorous tissues increased significantly with ANG mRNA expression ($r = 0.380$, $P = 0.001$) and ANG protein expression ($P < 0.01$). The ANG expression levels of cancer tissues were positively correlated with VEGF ($P < 0.01$) and the proliferation index of cancer cells ($P < 0.01$).

CONCLUSION: ANG is one of the neovascularization factors of HGC. ANG may work in coordination with VEGF, and promote the proliferation of HGC cells.

Key words: Angiogenin; Gastric carcinoma; Vascular endothelial growth factor; Angiogenesis

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INTRODUCTION

Primary gastric carcinoma is one of the most common malignant tumors in China. The mortality rate is as high as 25.53/100 000, accounting for 23.93% of all the deaths caused by malignant tumors[1]. Invasion and metastasis are the main cause for the death of cancer patients. On the other hand, invasion and metastasis of malignant tumors are closely related with angiogenesis of tumor tissue. Angiogenin (ANG) was originally isolated from serum-free supernatants of a cultured human colon adenocarcinoma cell line (HT-29) by Fett etcetera in 1985[2]. ANG possesses a ribonuclease activity[3]. Data have shown that in human colorectal cancer[4], pancreatic cancer[5], and other tumors, the expression of ANG is upregulated. However, the function of ANG in tumor angiogenesis is still unknown[6]. We used RT-PCR and immunohistochemistry to detect the expression of ANG, CD34 and vascular endothelial growth factor (VEGF) in 68 primary gastric carcinomas and the surrounding nontumorous tissues, analyzed their correlation with each other, and explored the functional role of ANG in angiogenesis and growth of gastric carcinoma.

MATERIALS AND METHODS

Materials

The study enrolled 68 human gastric carcinoma (HGC) patients (56 men and 12 women; Ages, mean ± SD, 60 ± 11 years) who underwent gastric surgical resection at The First Affiliated Hospital of Fujian Medical University between March and July of 2002. The specimens for RT-PCR included both cancer tissues and the surrounding nontumorous tissues. The specimens for immunohistochemistry were taken simultaneously. All specimens were 40 g/L formaldehyde-fixed, paraffin-embedded and sliced into 4 μm sections consecutively. None of these patients had preoperative radiative therapy or chemotherapy. All the diagnoses were confirmed by pathology reported by two pathologists. Clinical data of all the patients were reviewed. Histology type, lymph node metastasis stage and TNM stage of each case were determined according to WHO tumor classification standards (stomach cancer)[7]. Twenty-seven patients had

well-moderately differentiated adenocarcinoma, and 41 patients had poorly-undifferentiated adenocarcinoma. Fourteen patients were node-negative; 46 had first station lymph node metastasis, and 8 had second or distant lymph node metastasis. Twelve tumors were TNM Ⅰ, 25 tumors were TNM Ⅱ, 20 tumors were TNM Ⅲ, and 11 tumors were TNM Ⅳ.

Methods

RNA extraction: Specimens were taken within 30 min after resection, and were stored at -80℃. RNA was extracted from HGC tissue and corresponding nontumorous gastric tissue using Trizol extraction reagent kit (Gibco BRL, USA), according to the instructions of the manual. Concentration and purity of RNA were determined by spectrophotometric method. Integrity of RNA was determined by electrophoresis.

RT-PCR and analysis of the products: ANG primers were synthesized by the Bioasia Biotechnology Company using the method as described previously[10]. Each tube contained 5 × buffer 5 μL, 10 mmol/L dNTP 0.5 μL, DTT 1.25 μL, primers (sense, antisense) 4 μL, RNA template 1 μg, mix-enzyme 0.5 μL, DEPC solution, with a total volume of 25 μL (Titan One Tube RT-PCR system, Roche, Germany). RT-PCR reaction condition is as follows: at 58℃ for reverse transcription for 30 min, 94℃ incubation for 2 min to terminate reverse transcription, followed by 30 cycles at 94℃ for 30 s, at 52℃ for 30 s, and at 72℃ for 60 s; With a final extension at 72℃ for 7 min. RT-PCRs were run in a Bio-rad thermocycler. The results were analysed in a Bio-rad GelDoc-1000 system, relative to the levels of β actin as the control.

Immunohistochemistry: ANG multiclonal antibody 1:25 was purchased from American Santa Cruz Co. VEGF-C multiclonal antibody (ready to use) was provided by American Zymed Co. Monoclonal antibody of CD34 (QBEnd/10, ready to use) and monoclonal antibody of Ki-67 (MIB-1, 1:50) were supplied by American NeoMarkers Co. All operations were done according to the instructions of the manufacturers. Positive specimens were used as positive controls and PBS in substitution of the first antibody was used as a negative control at the same time.

ANG protein expression[8]**:** Cytoplasm of gastric carcinoma cells was stained brown. According to the staining intensity of the tumor cells, the staining was graded as (-), when staining was weaker than negative control; (+), for light staining; (++), for moderate staining; and (+++), for strong staining.

MVD of HGC tissues[9]**:** Gastric carcinoma vascular endothelial cells were stained brown. The isolated brown and yellow blood vessel endothelial cells or cell clusters in gastric carcinoma tissues were regarded as a single microvessel (Figure 1A). Areas with the highest microvessel densities were selected under 10 × microscopic magnification. Then the numbers of microvessels stained by CD34 antibody in 10 vision fields were counted under 400 × magnification (0.916 mm^2 per visual field), respectively. The average of 3 highest values was taken as MVD. Indistinguishable or indistinct cells were excluded.

VEGF protein expression: Membrane and cytoplasm of gastric carcinoma cells were stained rose. The staining intensity of the tumor cells was classified as: light staining (+), moderate staining (++), deep staining (+++).

Proliferation index of tumor cells: The nuclei of the positive cells were stained deep brown (Figure 1B). The number of positive cells among 1000 tumor cells was counted per slide and taken as the tumor cell proliferation index.

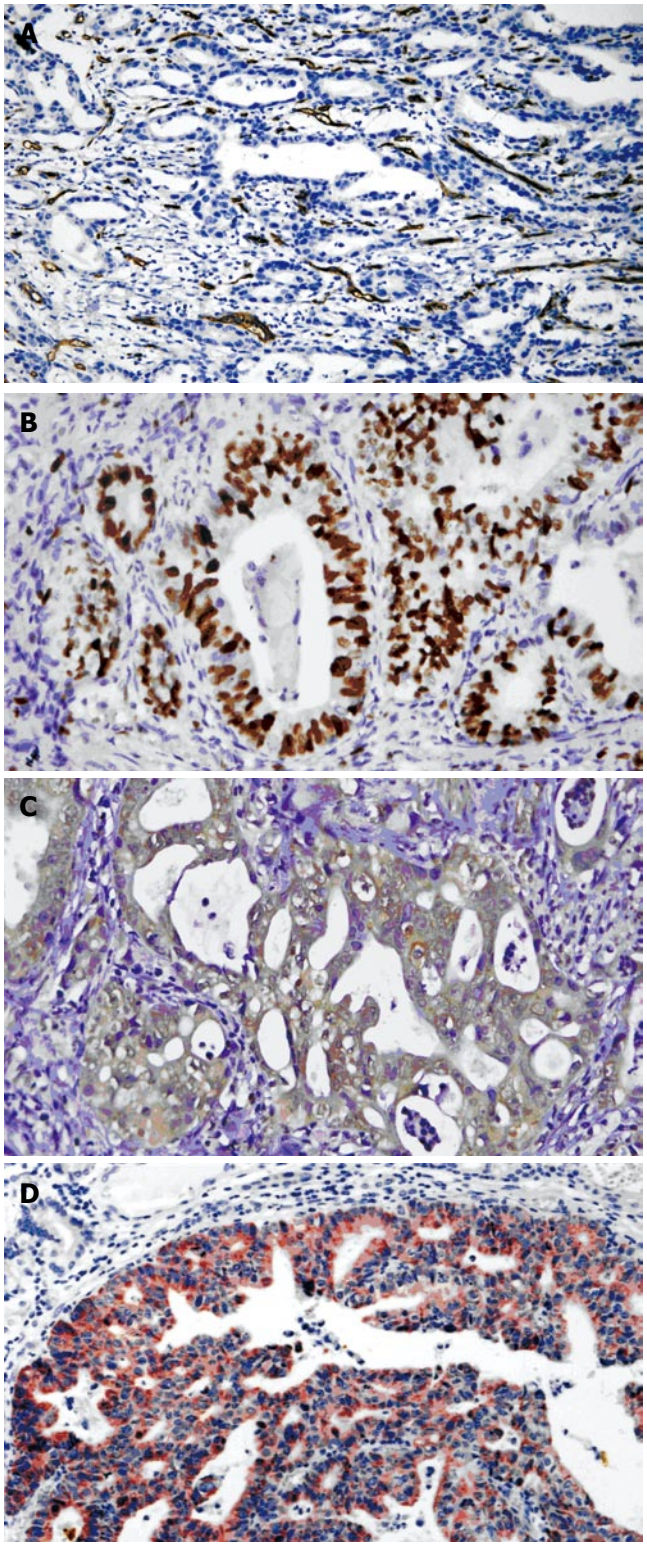

Figure 1 Expression of CD34, Ki-67, ANG and VEGF in gastric carcinoma tissue. **A**: CD34 (S-P × 200); **B**: Ki-67 (S-P × 400); **C**: ANG (EnVision × 400); **D**: VEGF (S-P × 200).

Table 1 ANG and ANG mRNA expression and clinicopathological characteristics of gastric carcinoma

Characteristics	*n*	ANG mRNA (mean ± SE)	ANG			
			+	++	+++	++++
Age (yr)						
< 60	37	1.727 ± 0.287	3	7	19	8
⩾ 60	31	2.634 ± 0.698	0	4	16	11
Site						
Cardia	25	2.866 ± 0.610	0	1	14	10
Corpus	10	1.310 ± 0.166	1	1	4	4
Antrum	25	2.366 ± 0.737	2	6	13	4
Others	8	1.704 ± 0.508	0	3	4	1
Differentiation						
Well-moderate	27	1.986 ± 0.247	1	2	15	9
Poor	41	2.242 ± 0.570	2	9	20	10
Depth of invasion						
Lamina and muscularis propria	12	1.384 ± 0.148	2	2	6	2
Visceral peritoneum	56	2.302 ± 0.429	1	9	29	17
Lymph node metastasis						
(-)	14	1.722 ± 0.186	1	1	9	3
(+)	54	2.245 ± 0.445	2	10	26	16
TNM stage						
I	12	1.756 ± 0.212	1	1	8	2
II	25	1.621 ± 0.273	2	3	10	10
III-IV	31	2.708 ± 0.739	0	7	17	7

Statistical analysis

Statistics software package was used for analysis. *t* test, analysis of variance, chi-square test and correlation analysis were used. $P < 0.05$ was taken as significance.

RESULTS

ANG mRNA expression in HGC tissues

ANG mRNA and its inner control β-actin were detected both in gastric carcinoma tissues and in corresponding nontumorous gastric tissues of all 68 cases. The RT-PCR products of ANG and β-actin were seen at 402 bp and 234 bp by gel electrophoresis (Figure 2). The expression of ANG mRNA was higher in 68 gastric carcinoma tissues (0.4822 ± 0.0943) than in corresponding nontumorous gastric tissues (0.2758 ± 0.0187, $P < 0.05$). Although ANG mRNA expression was higher in poorly differentiated carcinomas, in carcinomas invading visceral peritoneum, carcinomas with lymph node metastasis and TNM stage III-IV than in well-moderately differentiated adenocarcinoma, in carcinomas invading lamina and muscularis propria, carcinomas without lymph node metastasis and TNM stage I-II, no statistical difference was found (Table 1).

ANG expression in HGC tissues

Immunohistochemistry staining showed that ANG located at the cytoplasm of gastric carcinoma cells as brown particles (Figure 1C). Over 90% of the gastric carcinoma cells were stained positive. The ANG expression level had no correlation with the age of patients, location, histological type, lymph node metastasis, and clinical stage of the tumor (Table 1).

ANG expression and the MVD of tumor tissues

ANG mRNA expression ratio of HGC tissues and the corresponding nontumorous gastric tissues (T/N) was significantly correlated with the MVD of gastric carcinoma tissue. With the increase of ANG mRNA expression, MVD elevated also. A significant positive correlation was found between them ($r = 0.380$, $P < 0.01$). Also, with increase of ANG expression of gastric carcinoma tissues, MVD rose as well. The MVD of the ANG strong staining group (308.37 ± 25.57) was significantly higher than that of the negative group (196.00 ± 31.34) ($P < 0.01$).

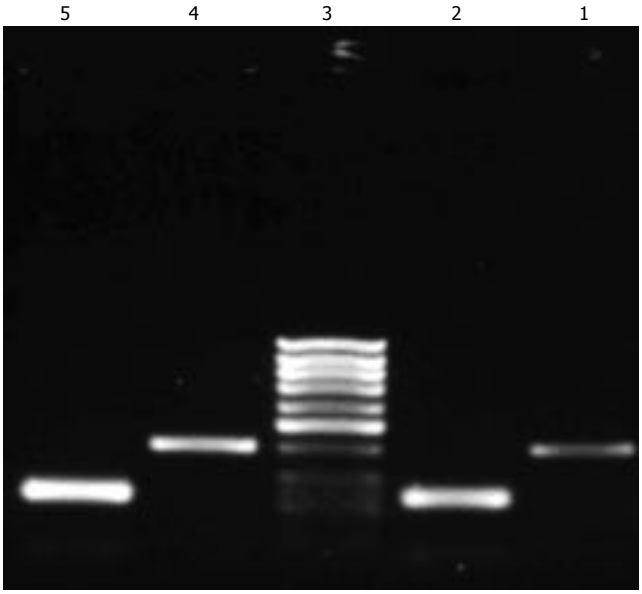

Figure 2 RT-PCR product electrophoresis. **1**: β-actin of gastric carcinoma tissue, 234 bp; **2**: ANG of gastric carcinoma tissue, 402 bp; **3**: molecular weight marker; **4**: β-actin of paired adjacent gastric mucosas, 234 bp; **5**: ANG of paired adjacent gastric mucosas, 402 bp.

Correlation between VEGF and ANG expression in HGC

The ANG expression in gastric carcinoma tissues was positively correlated with the expression of VEGF (Figure 1D) (Pearson correlation coefficient = 0.3490, $P < 0.01$).

Correlation between tumor proliferation index and ANG expression in HGC

The ANG expression levels of cancer tissues were positively correlated with the proliferation index of cancer cells ($P < 0.01$). The proliferation index of cancer cells of the group with strong ANG expression (579.58 ± 31.38) was much higher than that of the group with weak ANG expression (341.33 ± 84.01).

DISCUSSION

The up-regulation of expression of angiogenic factors in tumor tissue plays the role of switch-on in neoplastic angiogenesis. Research showed that hypoxia could induce up-regulation of ANG expression in human malignant melanoma, whereas it could induce down-regulation of ANG expression in normal melanocytes[10]. It has been shown that ANG mRNA expression is significantly higher in human colorectal cancer[4], pancreatic cancer[5], malignant melanoma[10], bladder carcinoma[11] and other tumors than in normal tissues. Our results are similar to these. The ANG expression levels of gastric

carcinoma tissues were significantly higher than that in the surrounding nontumorous gastric mucosas. Both ANG mRNA and protein expression levels had no significant correlation with the patients' age, location, histological type, lymph node metastasis, and clinical stage of the tumor[12]. Studies on pancreatic cancer[5], invasive breast carcinoma[13] and invasive cervical cancer[14] have obtained similar results. In malignant melanoma, ANG expression is upregulated around necrotic areas and invasive borders of tumors, while necrotic tissues do not express ANG. This suggests that hypoxia is the reason for the upregulation of ANG in gastric carcinoma tissues.

Tumor angiogenesis is the precondition of tumor development. Tumor MVD is recognized as an important index for judging tumor angiogenesis, which is a multi-step process. Under the effect of angiogenic factors, endothelial cells proliferate and migrate to form new vessels, and new blood vessel networks. This study showed a positive correlation between ANG expression levels and tumor vascularity evaluated by MVD, suggesting ANG contributes to the angiogenesis of HGC. In addition, previous studies have shown ANG contributes to the neovascularization of hepatocellular carcinoma[15], human colorectal cancer, and malignant melanoma[10]. ANG is one of the efficient angiogenic factors, and the only angiogenic factor with RNase activity[16]. There are two kinds of ANG-binding proteins on endothelial cells. One is actin, the other is a 170-kDa ANG-binding protein. Binding of ANG to cell surface actin causes activation of a cell-associated protease system and cell invasion. After the cells are activated, they migrate and invade the basement membrane, resulting in a decrease in the local cell density in the vicinity of the migrating cells, which may trigger the putative 170-kDa receptor gene expression in the remaining cells. These cells become ANG-responsive and divide to fill the space left by the migrating cells. The expression of the receptor gene is turned off when the gap is filled and cells become more dense. It may well be possible that the ANG-induced formation of a new capillary network is largely regulated by such cell density-dependent expression of the ANG receptor gene[3].

ANG is required for cell proliferation induced by various other angiogenic proteins including acidic and basic fibroblast growth factors (aFGF and bFGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF). Angiogenin-stimulated rRNA transcription in endothelial cells may thus serve as a crossroad in the process of angiogenesis induced by various angiogenic factors[17].

We found a positive correlation between ANG protein expression and VEGF expression in HGC tissues, suggesting ANG and VEGF may work in coordination in HGC angiogenesis. Asthmatic children have significantly higher levels of VEGF and angiogenin than healthy control children. A significant positive correlation exists between both angiogenic factors[18]. Moreover, a correlation has been found between VEGF and ANG levels during fetal development and in maternal diabetes[19]. Gonadotropin can not only stimulate the secretion of ANG by granulosa cells, but can also stimulate the secretion of VEGF by ovarian follicles. ANG and VEGF may also work together in the angiogenesis of corpus luteum[20]. Meanwhile, it was also found that hypoxia could induce the expression of VEGF[16]. Hypoxic induced factor-1 (HIF-1) binds VEGF oxygen sensitive enhancer under hypoxic conditions. Through the activation of c-Src, it promotes the transcription and expression of VEGF. Expression of ANG may be up-regulated in response to hypoxia within the tumor. A significant increase in the secretion and mRNA expression of ANG from both term placental explants and trophoblast cultures subjected to hypoxia *in vitro* was observed[21]. One study showed that ANG expression in invasive carcinomas is significantly positively correlated with HIF-1 alpha and the HIF-1 alpha target gene DEC-1[22]. Hypoxia can also induce upregulated expression of ANG in corpus luteum granulosa cells. This is related to two HIF binding sites on the ANG promoter[20]. Moreover, under hypoxic conditions, the ANG and VEGF secreted by renal proximal tubular epithelial cells may modulate angiogenesis and vascular remodeling in the renal *interstitium via* an increase in the production of HIF-1[23]. Accordingly, hypoxia in HGC tissues may stimulate ANG and VEGF expression through the HIF-1 pathway. ANG and VEGF may work coordinately in the angiogenesis of HGC.

We found that ANG expression levels were closely correlated with rapid growth and metastasis of malignant tumors. Pancreatic cancers with high serum ANG (sANG) levels had larger volumes than those to low sANG levels[5]. ANG expression in the gastric carcinoma tissues was significantly and positively correlated with the proliferation index of cancer cells. This suggests ANG could promote the growth of tumor cells. Studies have found that ANG can specifically combine with the leukemia cell line TF-1 and dose dependently promotes the growth of the leukemia cells[24]. ANG is constitutively translocated into the nucleus of HeLa cells where it stimulates rRNA transcription, ribosome biogenesis, proliferation and tumorigenesis[25].

In summary, ANG contributes to gastric carcinoma angiogenesis, and promotes the growth of tumor cells. Inhibition or blockage of the expression of ANG does not only reduce tumor angiogenesis, but also inhibits the growth of tumors directly. These have potential implications in tumor treatment.

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COLORECTAL CANCER

Prolonged exposure of colon cancer cells to the epidermal growth factor receptor inhibitor gefitinib (Iressa™) and to the antiangiogenic agent ZD6474: Cytotoxic and biomolecular effects

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Key words: Gefitinib; ZD6474; Colon cancer; Tyrosine kinase; Chemo-resistance

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Abstract

AIM: To analyze the biological effects of prolonged *in vitro* exposure of HT-29 and LoVo colon cancer cell lines to gefitinib (Iressa™), an inhibitor of epidermal growth factor receptor (EGFR) activity, and ZD6474, an inhibitor of both KDR and EGFR activities.

METHODS: Cells were treated with each drug for up to 2 wk using either a continuous or an intermittent (4 d of drug exposure followed by 3 d of washout each week) schedule.

RESULTS: In both cell types, prolonged exposure (up to 14 d) to gefitinib or ZD6474 produced a similar inhibition of cell growth that was persistent and independent of the treatment schedule. The effects on cell growth were associated with a pronounced inhibition of p-EGFR and/or p-KDR expression. Treatment with gefitinib or ZD6474 also inhibited the expression of EGFR downstream signal molecules, p-Erk1/2 and p-Akt, although the magnitude of these effects varied between treatments and cell lines. Furthermore, expression of the drug resistance-related protein ABCG2 was shown to significantly increase after 14 d of continuous exposure to the two drugs.

CONCLUSION: We conclude that long-term exposure of colon cancer cells to gefitinib and ZD6474 does not modify their cytotoxic effects but it might have an effect on sensitivity to classical cytotoxic drugs.

INTRODUCTION

Recently, a greater understanding of the molecular basis of cancer has fostered the development of rationally designed molecular-targeted therapies with agents that have been shown to prevent cell proliferation, differentiation and survival through the inhibition of receptor tyrosine kinases (TKs), such as epidermal growth factor receptor-1 (EGFR) and vascular endothelial growth factor receptors (VEGFRs)[1,2].

Drugs that specifically target these receptors act *via* inactivation of the tyrosine kinase function of EGFR receptors resulting in lack of recruitment and phosphorylation of several intracellular substrates. A major downstream signalling route involved in this process is the Ras-Raf-MAPK pathway finally leading to ERK1 and 2 inactivation[3,4]. Another important target in EGFR signalling is PI3K and the downstream AKT protein transducing signals to the cascade of survival and motility[5,6]. Recently, the relevant role of the oncosuppressor gene PTEN in uncoupling some of these signalling pathways and thus generating gefitinib resistance has also been stressed[7].

Gefitinib (Iressa) ia a well-known oral EGFR inhibitor that is able to reduce tumour growth and the formation of metastases in a range of human cancer cell lines and human tumour xenografts[8,9]. In the clinical setting, gefitinib monotherapy has demonstrated antitumour activity in patients with recurrent or refractory non-small-cell lung cancer[10,11] and it has been approved for clinical cancer treatment in several countries. ZD6474 is a novel, orally available antiangiogenic agent that selectively targets two

key tumour growth pathways by inhibiting VEGFR and EGFR tyrosine kinase activities. Preclinical studies have shown ZD6474 to be a potent inhibitor of VEGF-induced endothelial cell proliferation, tumour-induced angiogenesis and tumour growth[12].

Combining gefitinib or ZD6474 with other biological or cytotoxic agents has resulted in enhanced antitumour effects *in vitro* and *in vivo*[13-17]. Several ongoing clinical trials are therefore investigating the clinical efficacy of these targeting molecules when administered (1-21 or 1-28 d) in combination with agents such as taxols, gemcitabine, cisplatin, oxaliplatin, 5-FU and irinotecan [www.clinicaltrials.gov]. However, we have recently demonstrated that the effect of gefitinib used in combination with some cytotoxic drugs, can be schedule-dependent and have concluded that only extensive analysis of their main pathways of action could help in the design of an optimal multidrug therapy[13,15,16]. The great influence that schedules can have in combination treatments with TK-inhibitors and other cytotoxic drugs is also evinced by the fact that EGFR inhibitors can variously interact with proteins involved in resistance to some conventional cytotoxic drugs[18,19]. In particular, ABCG2 and mdr-P glycoprotein (P-gP), belonging to ATP-binding cassette (ABC) transporters, are involved in sensitivity to topoisomerase I inhibitors[20] and anthracyclines[21].

In vitro data analyzing the activity of TK inhibitors on cell growth , apoptosis induction or cell cycle and target modulation are mainly limited to very short term cell exposures to drugs, generally lasting no longer than 5 d[22-24]. Although such exposure times are useful to clarify the molecular mechanisms of action of this class of drugs, they do not account for some major cell mechanisms controlling the expression and function of EGFR receptors[25]. Some such mechanisms that are known to produce EGFR down-regulation are endocytosis, pH-sensitive dissociation, dephosphorilation by PTP1B, trafficking to the lysosome, *etc*.[26-29].

Data from *in vivo* studies on the effects of prolonged exposure to TK inhibitors are scant. An *in vivo* study on long-term exposure confirmed that TK inhibitors are able to reduce tumour growth when utilized alone or in association with other drugs[9,30] but at least one other study has demonstrated that tumour cell lines can develop resistance to gefitinib[31]. The only *in vitro* report analyzing the pharmacological and biological effects of prolonged exposure to an EGFR inhibitor supports the hypothesis that duration of cell exposure to such a drug is important in modulating its antitumour effect and synergism with other drugs[32].

The aim of this study was to investigate the cytotoxic and biomolecular effects of different gefitinib and ZD6474 long-term exposure modalities on colon cancer cell lines and, whether phosphorylation of their specific targets (EGFR and/or KDR), activity of downstream signalling molecules and multidrug resistance proteins were modified in an exposure time-dependent manner.

MATERIALS AND METHODS

Drugs and chemicals

Gefitinib and ZD6474 were provided by AstraZeneca Pharmaceuticals (Macclesfield, UK). Stock solutions were prepared at 20 μmol/L in dimethyl sulphoxide (DMSO) and stored in aliquots at -20℃. Further dilutions were made in F-12/HAM or McCoy's medium supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 50 000 U/L penicillin, and 80 μmol/L streptomycin.

Cell lines

Two colon cancer cell lines of human origin were used, LoVo and HT-29. Cells were routinely cultured in F-12/HAM (LoVo) or McCoy's (HT-29) medium, supplemented as above, in a humidified incubator at 37℃ with a 50 mL/L CO_2 atmosphere. Cells were trypsinized once a week with trypsin 0.25% ethylenediaminetetraacetic acid 0.02%, and the medium was changed twice a week. Doubling times were 20 ± 1 h for HT-29 cells and 24 ± 1 h for LoVo cells.

HPLC drug analysis

Gefitinib or ZD6474 (1-100 μmol/L) were incubated in F-12/HAM or McCoy's medium, supplemented as above, in a humidified incubator at 37℃ with a 50 mL/L CO_2 atmosphere for 1, 5 and 7 d, after which HPLC analysis was performed. The stock solutions of each drug in DMSO were used as controls. The HPLC consisted of an LC9010 system coupled with a UV detector (Varian Inc, Palo Alto, CA, USA). Purified drug was eluted from an Aspec Bond Elut-C2 column (Varian Inc) using a solution of triethylamine and acetonitrile (76:24 [v/v]) adjusted to pH 3.0 with 0.2 mol/L phosphoric acid.

Evaluation of cytotoxicity

Determination of the IC_{50} of gefitinib or ZD6474 was performed using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumbromide (MTT) assay. On d 1, 10 000 cells/well in a volume of 200 μL were added to 96-well plates. In each plate, one column contained cells not exposed to the drug (control), and five columns contained cells exposed to increasing concentrations of the drug. Each drug concentration was repeated in six identical wells. On d 2, gefitinib or ZD6474 (0.01, 0.1, 1, 10 and 100 μmol/L) was added allowing for different times of drug exposure (18 h to 3 d). Results were expressed as dose-effect curves with a plot of the fraction of unaffected (surviving) cells *versus* drug concentration. The IC_{50} was defined as the drug concentration yielding a fraction = 0.5 of affected (not surviving) cells, compared with untreated controls.

Cell growth inhibition following prolonged drug exposure

LoVo and HT-29 cells were exposed to gefitinib (0.12 μmol/L and 1.2 μmol/L, respectively) and ZD6474 (0.6 μmol/L and 5 μmol/L, respectively). These subtoxic concentrations induced 30% cell growth inhibition after 3 d of continuous drug exposure. Cell survival was determined by cell counts after 7 and 14 d of drug exposure, using either a continuous or intermittent (4 d with drug followed by a 3-d washout) schedule. Controls were untreated cells which, at a quite complete confluence, were counted, divided, and plated again at 70% of confluence. Results were expressed as percentage of cell survival compared with untreated controls.

Table 1 AUC and retention time of gefitinib or ZD6474 following incubation for 7 d at 37℃, by HPLC analysis

Drug	Medium	Concentration (μmol/L)	Retention time (min)	AUC
Gefitinib	DMSO	100	2.47 ± 0.03	1.30 ± 0.20
	Complete medium	100	2.52 ± 0.05	1.27 ± 0.16
ZD6474	DMSO	100	1.75 ± 0.02	0.88 ± 0.08
	Complete medium	100	1.71 ± 0.04	0.86 ± 0.10

Table 2 IC_{50} of gefitinib and ZD6474 in HT-29 and LoVo cells at various times of incubation

Exposure time	Gefitinib (μmol/L)		ZD6474 (μmol/L)	
	LoVo cells	HT-29 cells	LoVo cells	HT-29 cells
18 h	48.5 ± 2.5	> 100	16 ± 5.1	80 ± 4.8
1 d	29 ± 3.1	> 100	13 ± 2.6	59 ± 3.6
2 d	16.5 ± 1.5	> 100	8.2 ± 3.8	19 ± 1.8
3 d	7.3 ± 0.9	23.6 ± 4.1	3.5 ± 0.9	10 ± 0.4

Each experiment was conducted in triplicate.

Cell-cycle perturbation and apoptosis

LoVo and HT-29 cells were exposed to gefitinib (0.12 μmol/L and 1.2 μmol/L, respectively) and ZD6474 (0.6 μmol/L and 5 μmol/L, respectively) for 7 and 14 d, using either a continuous or intermittent (4 d with drug followed by a 3-d washout) schedule. Cells were then harvested, washed twice in ice-cold PBS (pH 7.4), fixed in 4.5 mL of 70% ethanol at -20℃ and washed once again in ice-cold PBS. After resuspension of the pellet in PBS containing 1 mg/mL RNase and 0.01% NP-40, cellular DNA was stained with 50 μg/mL propidium iodide (Sigma-Aldrich, St Louis, MO, USA). Cells were stored in ice for 60 minutes before analysis. Cell cycle and apoptosis determinations were performed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and data were interpreted using the ModFit software provided by the manufacturer.

Table 3 Cell cycle and apoptosis modulation after prolonged exposure to gefitinib or ZD6474 in HT-29 and LoVo cells

	Drug exposure	LoVo cells		HT-29 cells	
		Apoptosis (%)	G0/G1 (%)	Apoptosis (%)	G0/G1 (%)
Control		0	74.3 ± 1.3	0	69.5 ± 1.4
Gefitinib	1 wk continuous	10.1 ± 0.5	80.4 ± 1.5	7 ± 0.4	70. 7 ± 1.7
	2 wk continuous	23.2 ± 0.9	87.9 ± 1.3	20 ± 1.2	77.2 ± 2.0
	1 wk intermittent	13.8 ± 0.6	75.4 ± 1.4	6.5 ± 0.3	67.1 ± 1.4
	2 wk intermittent	29.7 ± 0.8	78.0 ± 2.1	18.3 ± 1.8	69.7 ± 1.9
Control		0	74.3 ± 2.1	0	69.5 ± 1.8
ZD6474	1 wk continuous	15.4 ± 0.6	77.6 ± 1.9	7 ± 0.9	71.7 ± 2.1
	2 wk continuous	30.3 ± 0.9	81.8 ± 2.3	15 ± 0.8	75.2 ± 2.4
	1 wk intermittent	18.3 ± 1.3	74.6 ± 2.1	7.3 ± 1.1	70.2 ± 1.8
	2 wk intermittent	29.6 ± 1.5	76.5 ± 1.5	16.4 ± 2.2	72.0 ± 1.7

Each experiment was conducted in triplicate.

Immunoprecipitation and Western blot analysis of targets after prolonged drug exposure

Expressions of EGFR, KDR, Akt, p-Akt, Erk1/2, p-Erk1/2, PTEN, ABCG2 and PgP were determined by Western blot analysis using β-actin as the standard protein. Expressions of p-EGFR and p-KDR were determined by immunoprecipitation and Western blot. Protein bands were quantified by densitometric analysis followed by analysis with Quantity One software (BioRad, Hercules, CA, USA). The primary antibodies used were anti-EGFR (clone 13) and anti-KDR (clone A-3) from BD Transduction Laboratories (San Diego, CA, USA); Anti-Akt, anti-p-Akt, anti-Erk1/2 and anti-p-Erk1/2 (clone E10) from Cell Signaling Technology (Beverly, MA, USA); Anti-ABCG2 (clone BXP-21) from Alexis Corporation (Lausen, Switzerland); Anti-PgP (clone F4) and anti-β-actin from Sigma-Aldrich; And anti-phosphotyrosine polyclonal antibody PY99 and anti-PTEN (A2B1) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibodies used were horseradish-peroxidase-conjugated anti-mouse and anti-rabbit from Amersham Pharmacia Biotech (Upsala, Sweden).

Statistical analysis

All the *in vitro* experiments were performed in triplicate, and results are expressed as the mean ± SD unless otherwise indicated.

RESULTS

Time-dependent drug stability

To verify gefitinib and ZD6474 stability in our experimental conditions, each drug was incubated in complete medium for 1, 5 and 7 d. The HPLC retention time and the area under the curve (AUC) for the peak of each drug were measured and compared to those of the same drugs in DMSO (standard). The HPLC retention times for the internal standards were 2.47 min for gefitinib and 1.75 min for ZD6474. Each drug showed the same retention time as its standard, irrespective of concentration, exposure time and the absence or presence of complete medium; the AUC of each peak was proportional to the drug concentration. These results confirmed the stability of gefitinib and ZD6474 in our experimental conditions. Table 1 shows the AUC and retention times of gefitinib and ZD6474 peaks obtained by HPLC after 7 d of drug exposure.

Drug-dependent cell growth inhibition and apoptosis

The IC_{50} values for gefitinib and ZD6474 in HT-29 and LoVo cells are shown in Table 2. A time-dependent reduction in IC_{50} was observed for both drugs, with the IC_{50} after 3 d of exposure being 4-8 times lower than after 18 h of exposure.

Treatment with gefitinib for 1 wk, according to a continuous or intermittent schedule, did not significantly affect LoVo cell growth, and 2 wk of treatment was necessary to show an inhibitory effect (Figure 1A). By contrast, HT-29 cell growth was inhibited by gefitinib at 1 wk and to an even greater extent at 2 wk. ZD6474 induced a progressive, exposure-dependent inhibition of cell growth in both cell lines, but HT-29 cells appeared to be more sensitive than LoVo cells (Figure 1B).

As expected, prolonged exposure of the cells to gefit-

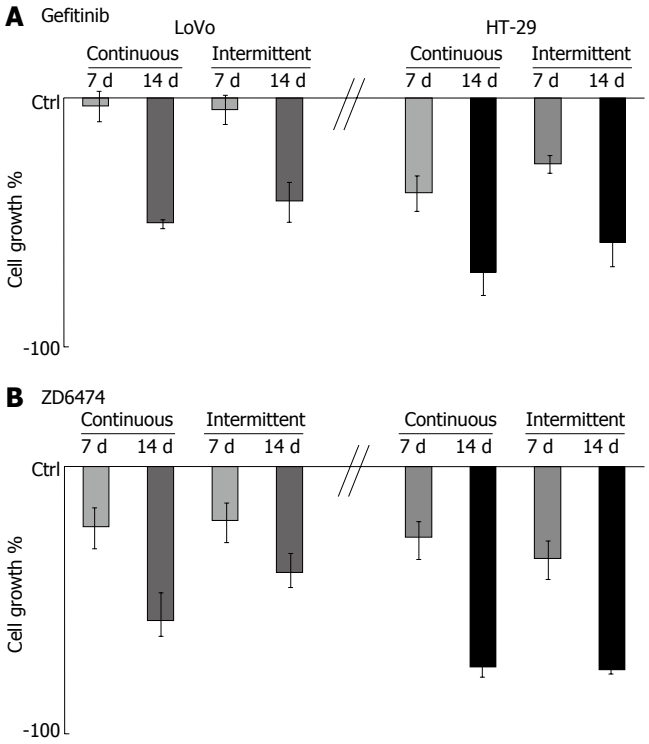

Figure 1 Drug-dependent cell growth inhibition. LoVo and HT-29 cells were incubated with gefitinib (0.12 and 1.2 μmol/L, respectively) or ZD6474 (0.6 and 5 μmol/L, respectively) for 7 and 14 d with continuous and intermittent exposure. Cell survival was determined by cell counts.

inib or ZD6474 produced an exposure-dependent increase in apoptosis (Table 3). This effect was observed with both the continuous and the intermittent treatment schedules. There was also a moderate accumulation of cells in the G0/G1 phase and this effect became more evident at 2 wk with continuous exposure (Table 3).

Modulation of TK receptor signal transduction pathways

The ability of gefitinib and ZD6474 to modulate their specific targets and downstream effectors was analyzed by measuring the expression levels of total and phosphorylated proteins.

TK receptor modulation: Prolonged exposure to gefitinib and ZD6474 of both cell lines, using either a continuous or intermittent treatment schedule, did not change the total amount of EGFR or KDR protein. HT-29 cells exposed to gefitinib for 7 and 14 d showed almost no detectable p-EGFR (Figure 2). In LoVo cells, gefitinib produced partial inhibition of p-EGFR that was appreciable only after 14 d of treatment. In both cell lines, ZD6474 almost completely inhibited p-KDR (approximately 98% inhibition compared with the control; Figure 2). ZD6474 also inhibited p-EGFR, with a greater effect in HT-29 cells than in LoVo cells.

EGFR signal transduction pathway modulation: Prolonged exposure to gefitinib and ZD6474 did not change the expression of Akt and Erk1/2 in HT-29 or LoVo cells. Gefitinib produced only modest effects on p-Akt but markedly decreased p-Erk1/2, the downstream effector of the proliferation pathway (Figure 3). Compared with the 7 d exposure, the magnitude of gefitinib-mediated

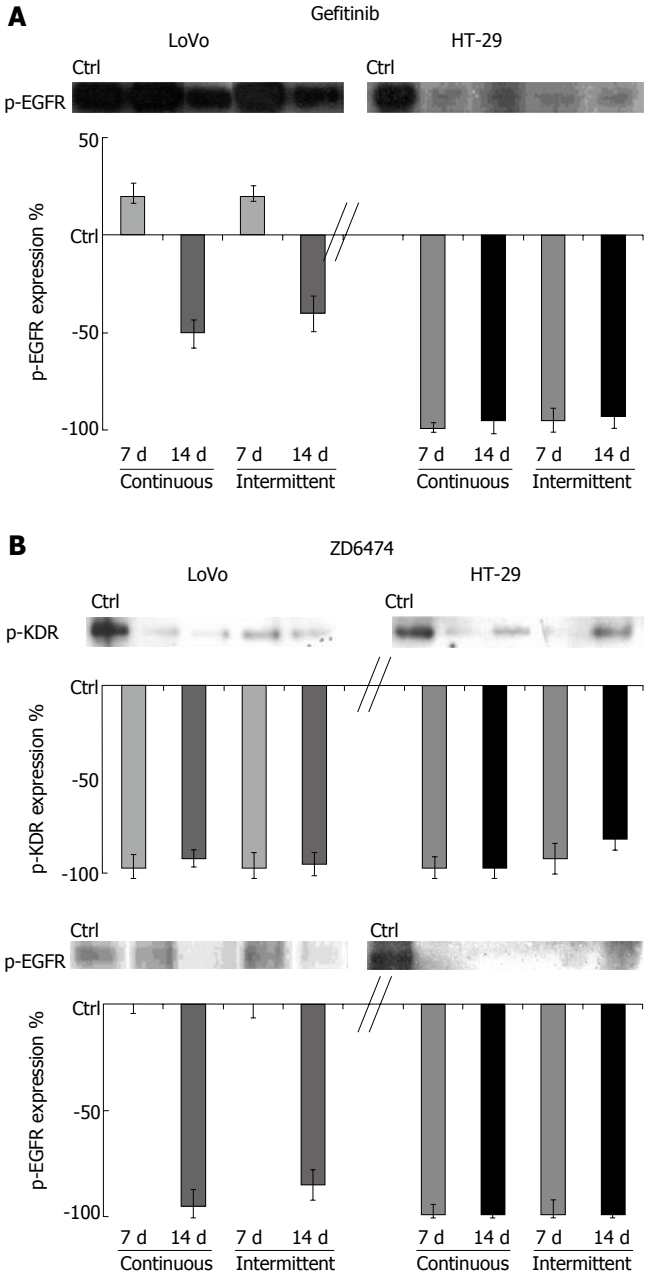

Figure 2 p-EGFR and p-KDR modulation after prolonged exposure to gefitinib or ZD6474. Cells were incubated with gefitinib or ZD6474 for 7 and 14 d with continuous and intermittent exposure. Drug-dependent modulation of p-EGFR (180 kDa) and p-KDR (195 kDa) was determined by immunoprecipitation followed by Western blotting. All data are shown relative to the baseline level (control = 0), which was similar after 7 and 14 d.

inhibition of p-Erk1/2 at 14 d was similar in the HT-29 cells, but had decreased in the LoVo cells.

Continuous or intermittent treatment with ZD6474 was associated with only a slight inhibition of p-Akt (Figure 3). ZD6474 induced a progressive and almost complete inhibition of p-Erk1/2 in HT-29 cells, but not in LoVo cells.

PTEN modulation: With a progressive increase in exposure time from 1 to 14 d, neither gefitinib nor ZD6474 modulated the total amount of PTEN in either cell line (data not shown).

Drug resistance induction

Preliminary analysis of the baseline expression levels of

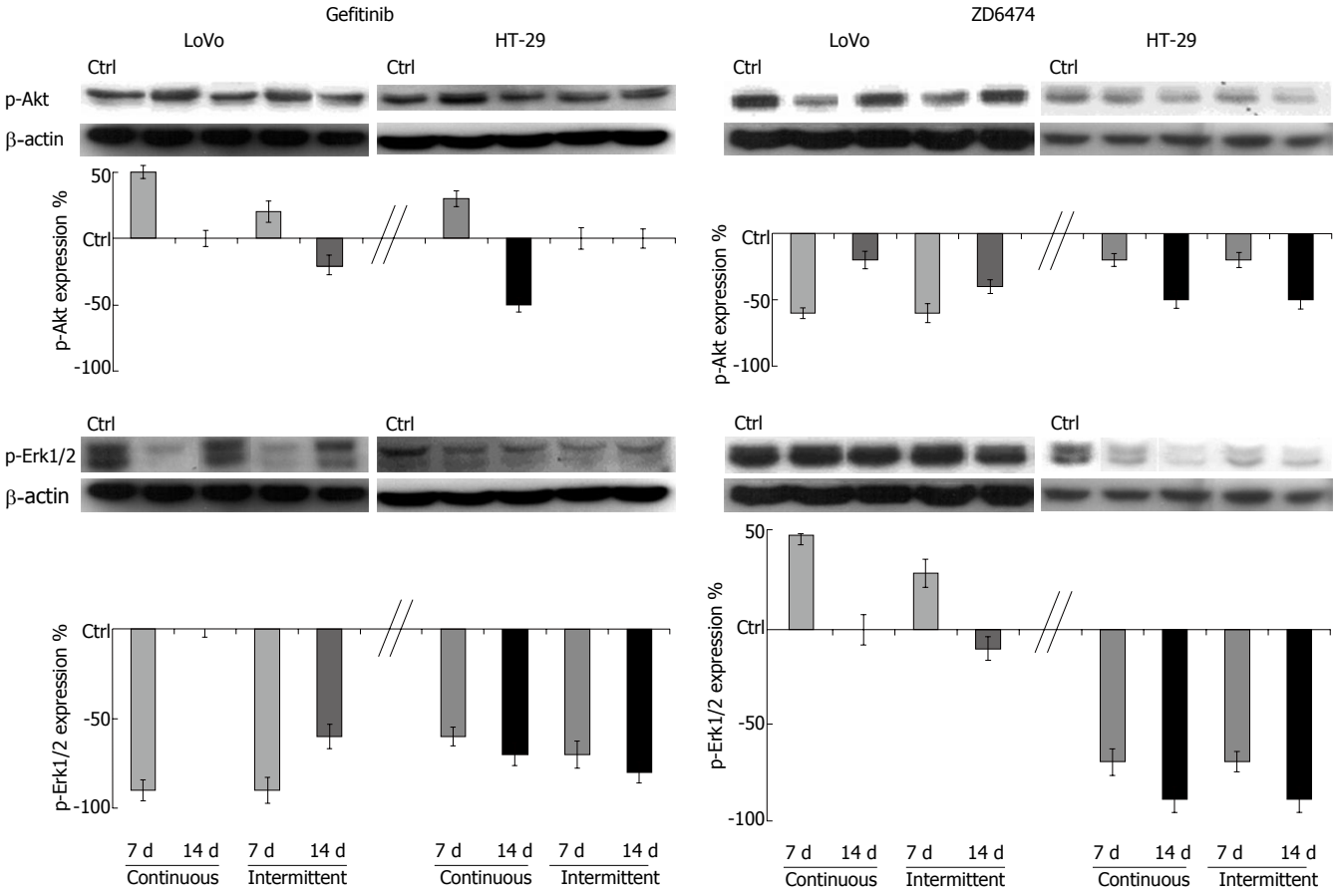

Figure 3 p-Akt and p-Erk1/2 modulation after prolonged exposure to gefitinib or ZD6474. Cells were incubated with gefitinib or ZD6474 for 7 and 14 d with continuous and intermittent exposure. Drug-dependent p-Akt and p-Erk1/2 modulation was determined by Western blotting. All data are shown relative to the baseline level (control = 0), which was similar after 7 and 14 d.

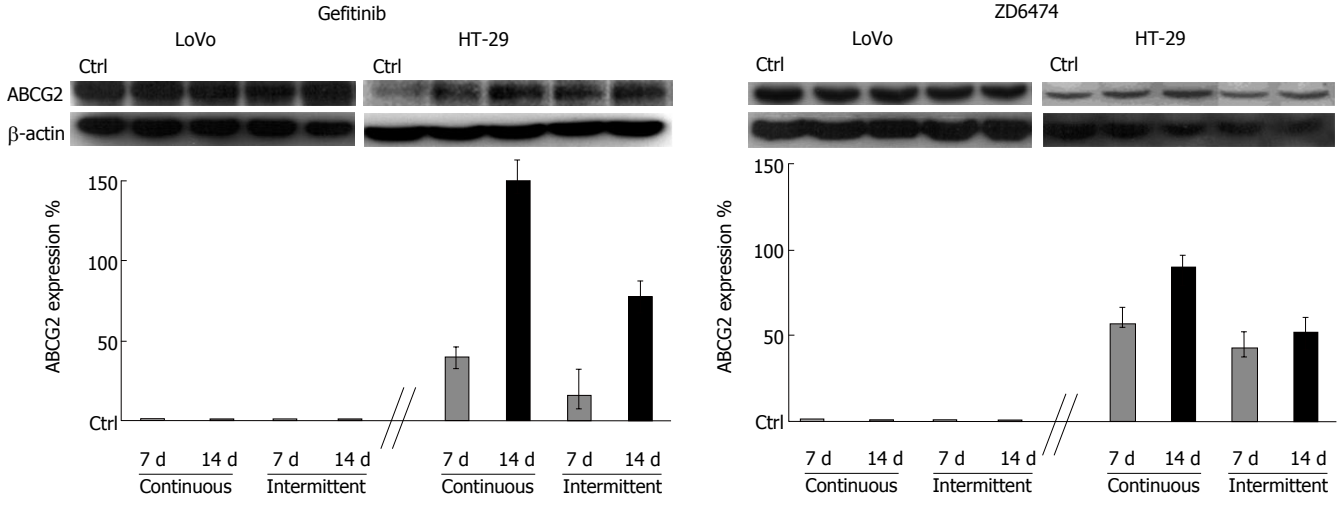

Figure 4 Increased ABCG2 expression after prolonged exposure to gefitinib or ZD6474. Cells were incubated with gefitinib or ZD6474 for 7 and 14 d with continuous and intermittent exposure. Drug-dependent increases in ABCG2 expression were determined by Western blotting. All data are shown relative to the baseline level (control = 0), which was similar after 7 and 14 d.

proteins related to drug resistance showed that ABCG2 was detectable in HT-29 and LoVo cells while P-gP was undetectable. LoVo cells also showed a higher level of ABCG2 than HT-29 cells. Interestingly, both gefitinib and ZD6474 exhibited time exposure-dependent increase in ABCG2 expression only in HT-29 cells; this effect became evident after 5 d of drug exposure (data not shown) and was progressive until 14 d (Figure 4). Stimulation of ABCG2 expression was more evident after continuous exposure to gefitinib or ZD6474 (150% and 90% increase in ABCG2 expression, respectively) than after intermittent exposure (80% and 50% increase compared with controls, respectively).

DISCUSSION

Analysis of the biomolecular effects that the TK inhibitors, gefitinib and ZD6474, can have on tumour cells after prolonged drug exposure is instrumental in leading to optimization of their use in monotherapies and in combination with other biological or conventional cytotoxic drugs[10,11,33,34]. Several cell effects have been demonstrated to depend directly on the modality and duration of cell exposure to these drugs; these effects include receptor expression, function of signal transduction, modulation of drug resistance proteins, etc. Nonetheless, and in spite of the clinical modalities of administration of these drugs, *in vitro* studies describing the inhibitory effect of gefitinib or ZD6474 on cell growth have considered only short drug exposures of 1-5 d[22-24].

In our study, we assessed the effects of prolonged exposure to the TK inhibitors, gefitinib and ZD6474, on cell viability and on their specific molecular targets by directly monitoring the modulation of the phosphorylated form of EGFR and of the two effectors, Akt and Erk1/2, that are important in the cell survival and proliferation pathways, respectively.

Two weeks exposure to gefitinib resulted in up to 70% cell growth inhibition and no apparent differences between the continuous and the intermittent treatment schedules. Moreover, as already reported for erbitux[32], gefitinib inhibited the phosphorylated forms of the receptor p-EGFR and of the downstream effector p-Erk1/2 involved in the proliferation pathway. The effects of gefitinib on p-Akt were less dramatic and appeared to be cell line-specific.

Comparison of these results with those obtained after short-term drug exposure[13] highlighted differences in the molecular effects produced by different drug schedules. Although a high rate of cells died after 2 wk of drug exposure in our investigation, cells tried to escape the attack by an exogenous agent by further modulating the survival and proliferation pathways. These findings suggest that a combination of Gefitinib and other PI3K/Akt pathway inhibitors, such as mTor inhibitor, may produce a more powerful synergistic effect.

ZD6474 proved to be able to inhibit the growth of our two colon cancer cell lines, thus confirming similar effects previously observed in GEO cells[23]. In both cell lines, the ZD6474-mediated inhibition of growth was associated with almost complete inhibition of p-KDR and p-EGFR, as well as a slight inhibition of p-Akt. In contrast, the modulation of p-Erk1/2 by ZD6474 was evident in HT-29 cells only.

Another aspect we considered was the effect on the expression of the tumour suppressor PTEN. Unlike Nagata, who reported PTEN modulation after 1 hour of exposure to the anti-ErbB2 antibody, trastuzumab[35], no appreciable modulation of this protein was observed in our study after short or long, continuous or alternate drug exposures. Our conclusion was that 14 d of drug exposure may be long enough for recovery of the possibly transient and short-term modulation of this gene potentially occurring in the first few hours after treatment with TK inhibitors.

Tyrosine kinase inhibitors have shown the potential to modulate cytotoxic drug resistance, through an interaction with ABCG2 and PgP[18,19]. In our study, ABCG2, which is involved in camptothecin resistance[20], was detectable in both HT-29 and LoVo cells while PgP, which is involved in anthracycline resistance, was not detectable in either cell line. The increased ABCG2 expression, following exposure to gefitinib or ZD6474, was cell line-specific. The HT-29 cells showed a progressive exposure-related increase of 100%-150%, while expression in the LoVo cells was unaffected. The increased expression of ABCG2 in HT-29 cells was schedule-dependent, and it was higher with continuous than with intermittent incubation. Our results seem to be in contrast with those obtained by Nakamura[19] but the experimental conditions used were completely different. Nakamura used a short time (15 min) to show a gefitinib-dependent increase in topotecan accumulation in transfectant cells, overexpressing ABCG2, and 4 d of gefitinib plus cytotoxic drugs exposure to led to reversal of drug resistance. In our experiments the ability of gefitinib to enhance ABCG2 expression proved to be a late effect of the drug and may account for the antagonism between Topoisomerase-I inhibitors and TK-inhibitors in HT-29 cells induced by a pre-exposure to gefitinib for 5 d[13]. Moreover, the cell-line-specific effects of gefitinib and ZD6474 on drug-related proteins may provide an explanation for the different results. Wakeling and Ciardiello obtained[30,31] when analyzing the onset of drug resistance *in vivo* after long-term drug intake.

In conclusion, our investigation studied, in an *in vitro* model of colon cancer, some crucial points related to the clinical use of these TK inhibitor drugs. Evidence has emerged that long term use (maximum 14 d) of these drugs does not lead to a loss of cell activity and intermittent or continuous exposures to these drugs do not produce significantly different toxic effects. The main signal transduction steps for TK receptor pathways were also studied and the findings demonstrated that the effects of these drugs may be highly cell-line-specific (HT-29 *vs* Lovo), drug-dependent (gefitinib *vs* ZD6474) and schedule-related (continuous *vs* intermittent), thus indicating that a predictive factor for these drugs cannot be easily identified or broadly applicable. The data concerning the modulation of drug resistance related proteins in tumour cells treated with long-term drug exposure are even more interesting. ABCG2 expression was shown to be induced by these drugs in a cell-line- specific and schedule-dependent manner. This evidence should suggest caution in choosing exposure times when combining these drugs with Topoisomerase-I inhibitors in particular. The findings of this study can be instrumental in the implementation of future *in vitro* and clinical studies on these drugs.

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COLORECTAL CANCER

BRAF, K-ras and BAT26 mutations in colorectal polyps and stool

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Abstract

AIM: To assess the feasibility of using BRAF, K-ras and BAT26 genes as stool-based molecular markers for detection of colorectal adenomas and hyperplastic polyps (HPs).

METHODS: We applied PCR-SSCP and direct sequencing to detect BRAF mutations of polyps and paired stool samples. Primer-mediated restriction fragment length polymorphism (RFLP) analysis and mutant-enriched PCR were used in detection of K-ras mutations of polyp tissues and paired stool samples respectively. BAT26, a microsatellite instability marker was examined by detection of small unstable alleles in a poly (A) repeat.

RESULTS: No genetic alterations were detected in the 36 colonoscopically normal patients in either tissues or stools. BRAF, K-ras and BAT26 mutations were found in 4 (16%), 10 (40%) and 3 (12%) of 25 adenoma tissues and among them, 75%, 80% and 100% of patients were observed to contain the same mutations in their corresponding stool samples. In HPs, mutations of BRAF and K-ras were detected in the tumor DNA of 2 (11.1%) and 8 (33.3%) of 18 patients respectively, all of whom had identical alterations in their stools. Taken together, the three genetic markers detected 15 (60%) of 25 adenomas and 8 (44.4%) of 18 HPs. The sensitivity of stool detection was 80% for adenomas and 100% for HPs with an overall specificity of 92% for adenomas and 100% for HPs.

CONCLUSION: BRAF, K-ras and BAT26 genes have the potential to be molecular markers for colorectal adenomas and HPs, and can be used as non-invasive screening markers for colorectal polyps.

Key words: Hyperplastic polyps; Adenomas; BRAF; Stool-based colorectal cancer diagnosis

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies in the world and it is a disease that can be easily cured either by surgery or by endoscopic excision when diagnosed at an early stage[1]. It has been generally accepted that a majority of CRC develop through a well-defined adenoma-carcinoma sequence in which multiple genetic changes are involved in this pathway that is known as chromosomal instability pathway. K-ras gene is known to play an important role along this pathway in transitioning from early to intermediate adenomas[2]. However, increasing evidence accumulates that a subset of CRC arises *via* the hyperplastic polyp (HP)-serrated adenoma (SA)-carcinoma sequence that is associated closely with microsatellite instability (MSI) positive colorectal carcinomas[3-5]. These polyps are usually large and/or multiple and/or located in the proximal colon[6,7]. APC mutation is not involved in this pathway whereas K-ras and BRAF mutations are frequently observed in these polyps with MSI[8,9]. Both BRAF and K-ras are proto-oncogenes that interact in tandem in the RAS-RAF-MEK-ERK-MAP kinase signaling pathway, which plays an important role in the control of cell differentiation, proliferation, survival, and apoptosis[10].

HPs have long been regarded as safe lesions without neoplastic potential[11]. However, this view has been changed due to the discovery of the HP-SA-carcinoma sequence. Now it is believed that adenomas as well as some types of HPs are precursors of sporadic CRCs that may eventually develop into adenocarcinomas[2,5]. So detection and surveillance of these premalignant polyps may be of great importance in reducing the incidence of colorectal cancer. BRAF and K-ras mutations have been frequently observed in both adenomas and HPs[8-10]. This prompted us to detect colon polyps through analysis of these genes in stool DNA. In this study, we detected mutational activation of the K-ras and BRAF genes and along with an MSI marker, BAT26 in patients with adenomas or HPs, and

performed a pair comparison between tumor tissue and stool sample in individual patients to assess the feasibility of using these genes as molecular markers for colon polyps.

MATERIALS AND METHODS

Patients and samples

We recruited 79 patients who underwent colonoscopy for various reasons at the 2nd Affiliated Hospital of Harbin Medical University from June 2004 to March 2005. These included 36 control patients without neoplasms, 18 patients with hyperplastic polyps and 25 patients with adenomas. Diagnosis was histologically confirmed. The mean age of control patients was 49.6 years compared with 51.6 years for patients with HPs and 56.5 years for patients with adenomas. Of the 43 polyps, 34 were from males and 9 were from females. Twenty-seven were from the left colon and 16 were from the right colon. The size of these polyps ranged from 3 to 50 mm in maximal dimension (mean = 5.28 mm for HPs and 18.7 mm for adenomas). Patients who had familial adenomatous polyposis or hereditary nonpolyposis colon cancer, and inflammatory bowel diseases were excluded.

Fresh stool specimens were collected from patients prior to colonoscopy. All the patients were given detailed oral and written instructions for stool collection. Stool samples were frozen immediately at -20℃ after collection and transferred to -80℃ for permanent storage within 24 h.

With the informed consent of all patients and approval of the ethics committee, paired bioptic polyp tissues were obtained during polypectomy. One part of the tumor was snap frozen in liquid nitrogen and stored at -80℃ until the extraction of nucleic acids. Another part was fixed in formalin and paraffin-embedded for diagnosis.

DNA extraction

DNA was isolated from stool samples by means of the QIAamp DNA stool mini kit (QIAGEN, USA) and from snap frozen tissues using Trizol reagent (Invitrogen, Life Technologies, USA) according to the manufacturer's instructions.

Mutational analysis and sequencing of the BRAF gene

To detect possible sequence alterations in BRAF, we performed nonisotopic single-strand conformational polymorphism (SSCP) analysis. The complete sequence of exon 15 of the BRAF gene was amplified from 50 ng of genomic DNA using the primers described previously[9]. PCR was carried out for 40 cycles with initial denaturation at 95℃ for 5 min, followed by 95℃ for 40 s, 55℃ for 40 s, and 72℃ for 40 s in a reaction volume of 25μL. The PCR products were then separated on 8% nondenaturing polyacrylamide gels (29:1) with or without 5% glycerol, and electrophoresis of the gels was carried out at 4℃-8℃. The gels were then fixed in 10% acetic acid, silver stained in a freshly prepared silver nitrate (0.1%) and developed in 3% sodium carbonate with 0.05% formaldehyde and 0.2% sodium thiosulphate.

All PCR products showing mobility shifts on SSCP were reamplified and purified, and evaluated by direct sequencing with an ABI 3700 DNA sequencer (Applied Biosystems).

Primer-mediated RFLP analysis

The mutations at codons 12 and 13 of K-ras gene in tumor samples were screened by primer-mediated restriction fragment length polymorphism (RFLP) analysis as described[12]. Human placental DNA (Sigma, USA) was used as a wild type control. Colorectal carcinoma DNA with known mutations at K-ras codon 12 (GGT to GTT) and at codon 13 (GGC to GAC) was used as mutant controls.

Mutant-enriched PCR

Mutant-enriched PCR was used to analyze the mutational status of the K-ras gene in stool samples. The procedure was the same as described[12] with the only exception that 5 U of BstNI or Bgl I (NEB, Beijing) was used for the digestion, and the reaction was completed overnight. Final products were separated on 4% agarose gel and visualized by ethidium bromide staining. The same positive and negative controls were used as in PCR-RFLP analysis.

MSI analysis

The MSI marker used was BAT-26, a mononucleotide repeat that by itself is a very good measure of generalized instability. PCR reactions were performed with the specific primers reported previously [13]. PCR products were loaded on 8% polyacrylamide/ 7 mol/L urea DNA-denaturing sequencing gels and silver stained as described above.

Statistical analysis

Differences between groups were assessed by × 2 test and Fisher's exact test. All P values were two sided. Factors with $P < 0.05$ were considered statistically significant. The 95% confidence intervals (CI) were determined based on the exact binomial distribution.

RESULTS

Twenty-five sporadic adenomas and 18 hyperplastic polyps were collected and analyzed for the alterations of K-ras, BRAF and BAT26 genes. The median age of the patients with HPs was 56 years (range: 28-70) compared with 61.8 years for patients with adenomas (range: 40-74). Thirty-six stool samples from control patients were also detected.

Adenomas

Mutations of exon 15 of BRAF and codons 12, 13 of K-ras genes were analyzed, as these cover most of the mutation hot spots known of the two genes. The alterations of BRAF, K-ras and BAT26 genes in adenomas and their relationship with clinico-pathological characteristics are shown in Table 1.

Of 25 tumor samples detected, 15 [60%, 95% CI: 39%-79%] were found to have at least one alteration in BRAF, K-ras or BAT26 genes. BRAF mutations were identified in 4 (16%, 95% CI: 5%-36%) cases, all at nucleotide position 1799 with T-A transversions (V599E) as confirmed by direct sequencing (Table 1, Figure 1). No significant correlation was found between BRAF mutations with

Table 1 Genetic analysis of adenomas and HPs compared with clinical-pathological characteristics

	n	BRAF *n* (%)	K-ras *n* (%)	BAT26 *n* (%)	All markers *n* (%)
Adenomas	25	4 (16)	10 (40)	3 (12)	15 (60)
Gender					
M	19	3 (15.8)	7 (36.8)	2 (10.5)	
F	6	1 (16.7)	3 (50)	1 (16.7)	
Age (yr)					
< 60	14	3 (21.4)	7 (50)	3 (21.4)	
⩾ 60	11	1 (9.1)	3 (27.3)	0	
Location					
Distal	16	2 (12.5)	8 (50)	1 (6.3)	
Proximal	9	2 (22.2)	2 (22.2)	2 (22.2)	
Size (mm)					
< 10	5	1 (20)	2 (40)	0	
⩾ 10	20	3 (15)	8 (40)	3 (15)	
HPs	18	2 (11.1)	6 (33.3)	0	8 (44.4)
Gender					
M	15	2 (13.3)	5 (33.3)	0	
F	3	0	1 (33.3)	0	
Age (yr)					
< 60	12	2 (16.7)	5 (41.7)	0	
⩾ 60	6	0	1 (16.7)	0	
Location					
Distal	11	1 (9.1)	4 (36.4)	0	
Proximal	7	1 (14.3)	2 (28.6)	0	
Size (mm)				0	
< 10	15	0	4 (26.7)	0	
⩾ 10	3	2 (66.7)[1]	2 (66.7)	0	
Number of HP					
SP	4	1 (25)	1 (25)	0	
MP	14	1 (7.1)	5 (35.5)	0	

Group HPs included the individuals with only hyperplastic polyps. If an individual had multiple polyps and if any one of the polyps exceeded 10 mm in diameter, it was regarded as ⩾ 10 mm in size. SP: Single polyp; MP: Multiple polyps. Proximal tumors were defined as cecum through transverse colon; tumors in the splenic flexture, descending, and sigmoid colon were defined as distal. [1]Fisher's exact test, $P = 0.01$.

Table 2 BRAF mutations in colorectal adenomas and HPs with BAT26 status

	No of samples	No of BRAF mutation (%)
Adenomas	25	4 (16)
BAT26 (+)	3	2 (66.7)[1]
BAT26 (-)	22	1 (4.5)
HPs	18	2 (11.1)
BAT26 (+)	0	0
BAT26 (-)	18	2 (11.1)

[1]Results from Fisher' exact test, BAT26 (+) *vs* BAT26 (-), $P = 0.028$.

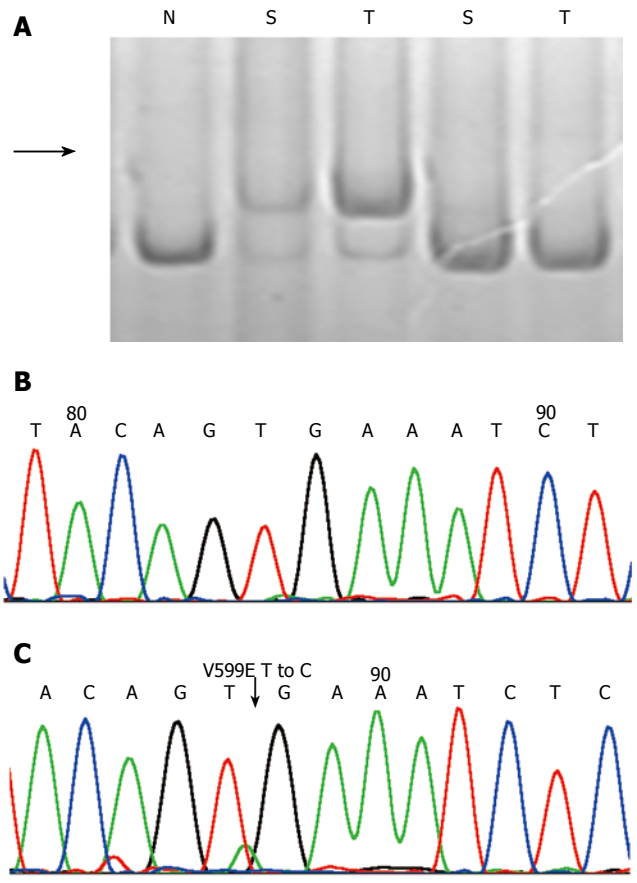

Figure 1 Example of BRAF mutation analysis in adenoma tissues (T) and paired stool (S) samples. **A**: SSCP analysis; **B**: Direct sequencing showing the wild type BRAF; **C**: BRAF V599E mutation; N: normal. Arrows indicate the new band (**A**) and the mutation site (**C**).

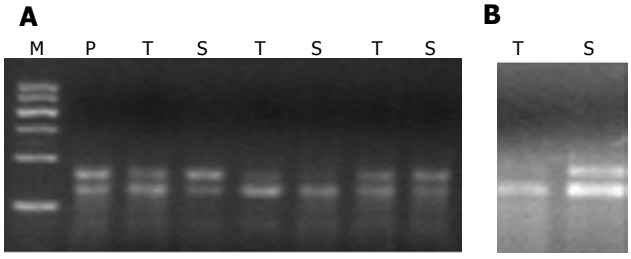

Figure 2 Analysis of K-ras mutations at codon 12 (**A**) and codon 13 (**B**) in adenoma tissues (T) and paired stool (S) samples. Fragments of 157 bp indicate mutations, and fragments of 128 bp(**A**) or 125 bp (**B**) respectively represent wild-type alleles. M: 100 bp ladder marker; P: Positive control; **B** shows the result that a mutation at codon 13 of K-ras in stool DNA was not observed in tumor counterpart.

patient's gender, age, location of tumor and tumor size (Table 1). The identical V599E mutations of the BRAF gene were also observed in 3/25 (12%, 95% CI: 3%-31%) of fecal samples with a 75% agreement between tumor and stool.

In total, 3/25 (12%, CI: 3%-31%) of tumor DNAs showed BAT26 alterations (Table 1) and the same mutations were also observed in all the 3 paired stool samples. Two of the 3 samples with a BAT26 alteration also harbored a BRAF mutation that showed a close relationship between BRAF mutation and MSI (66.7% *vs* 4.5%, $P < 0.05$, Table 2). All 3 BAT26 positive samples were found in patients younger than 60 year and in adenomas larger than 10 mm in size but of no statistical significance. Also no significant relationship was found with respect to patient's gender and tumor site.

Among 25 tumor samples analyzed for K-ras mutations at codons 12 and 13, 10 (40%; 95% CI: 21%-61%) samples were found to carry a mutation, 8 of which were at codon 12 and 2 at codon 13 (Table 1, Figure 2). Mutations of the K-ras gene in corresponding stool samples were detected by mutant-enriched PCR[12]. Of 25 stool samples

analyzed, K-ras alterations were observed in 9 (36%, 95% CI: 18%-58%) patients, 7 at codon 12 and 2 at codon 13. Eight of 10 stool DNAs had a mutation that matched exactly with the results of the corresponding tumor analysis (sensitivity: 80%, 95% CI: 44%-97%) whereas there was one case in which a K-ras mutation at codon 13 was observed in stool DNA but could not be detected in the corresponding tumor (specificity: 94%, 95% CI: 71%-100%, Figure 2). None of the tumor or stool samples with V599E carried a K-ras mutation.

To sum up, the three genetic markers detected 13/25 (52%) of stool samples of adenoma patients. The overall sensitivity of stool analysis using these three genes was 80% (12/15, 95% CI: 52%-96%) with a specificity of 92% (12/13, 95% CI: 64%-100%).

Hyperplastic polyps and normal controls

Eight (44.4%, 95% CI: 22%-69%) of the 18 HPs were found positive in either the BRAF or K-ras gene while no alterations were found in the BAT26 gene (Table 1). In corresponding stool samples, the same mutations were identified in all the 8 samples whose tumors contained the alterations with a 100% agreement between tumors and stools. No alterations were found in the other 10 samples. The specificity was also 100%. BRAF mutations were more common in polyps larger than 10 mm in size ($P < 0.05$, Table 1). BRAF and K-ras mutations were also mutually exclusive. No alterations were found in any of the three markers tested in 36 control DNAs of fecal origin.

DISCUSSION

In this study, we assessed the use of genetic alterations of BRAF, K-ras and BAT26 genes as stool-based molecular markers for colon adenomas. To our knowledge, this is the first time to show the results of stool-based gene detection of colorectal hyperplastic polyps.

We identified 16% of BRAF mutations and 40% of K-ras mutations in adenoma tissues. The results are similar to those reported previously[10]. However, in HPs, we observed a relatively lower incidence of BRAF mutations and a higher incidence of K-ras mutations than other studies [8,9]. Chan[8] reported a 36% BRAF mutation rate in HPs and Yang[9] reported an even higher mutation rate of 69.6%, but our result was only 11.1%. The incidence of K-ras mutations was 18% and 20% respectively by Chan[8] and Yang[9] but ours was 33.3%. This difference may be due to the small sample numbers in our study. The only form of BRAF mutation observed in our study was V599E. It is the most common mutation identified to date[8-10]. This missense mutation has been proven to maximally activate kinase activity of the BRAF protein and mutated forms of BRAF can transform NIH3T3 cells[14]. It is obvious that this variant has a strong functional selection for growth advantage. The high incidence of mutually exclusive mutations of BRAF and K-ras in both adenomas and HPs supports the previous hypothesis that activations of both genes are an early event in tumorigenesis of CRC[8-10].

BAT26 alteration was observed in 3/25 (12%) of adenomas, two of which also harbored a BRAF mutation. This close relationship between BRAF and MSI status agrees well with the findings in sporadic CRC[10]. By contrast, no BAT26 alteration was found in HPs. High level MSI was more often found in tumors located in the right colon, and most of our HPs were located in the left colon. However, several other studies have also reported a low MSI in HPs and serrated adenomas[15].

It is interesting to note that although not significant, in our study, patients with > 60 years of age had a tendency towards a lower prevalence of any mutation in both the adenoma and HPs groups. This may be due to the small sample numbers detected in our study, and larger sample analysis is needed to prove this phenomenon.

Based on our findings in the adenomas and HPs, we tried to use these three genes as stool based molecular markers for colon adenomas and HPs. Of the 15 adenomas that showed positive results, 12 (80%) samples have been detected to be positive in corresponding stool DNA, with a specificity of 92%. This result is quite comparable with the findings reported previously[16-20], in which DNA panel targeted mutations at K-ras, APC and p53 genes as well as BAT26 and long DNAs were used. But our detection seemed to be more convenient. First, mutation at the BRAF V599E hotspot is relatively simple to detect using SSCP and the sequencing method used in the present work (Figure 1). Second, the entire sequence of BRAF exon 15 is rather short, about 250 bp long, which can be amplified effortlessly, even in the stool DNA (data not shown).

In our study, we have detected a K-ras mutation in one stool DNA that could not be observed in corresponding adenoma tissue DNA. This is not caused by the different detection method we used in stools and tissues, because we analyzed this tissue DNA again using mutant-enriched PCR as in stool DNA, but still no mutation was found. This disparity might be due to other polyps present in the colon of this patient which we failed to detect through colonoscopy or because other neoplasms outside the colon were present in this patient that we did not know, since K-ras mutations have been detected in patients with pancreatic diseases[21].

In addition to adenomas, we also analyzed the DNA panel in patients with HPs. Our results showed that 44.4% of stool samples carried a mutation of either the BRAF or K-ras gene. All the mutations found in HPs could also be detected in corresponding stool DNA with a specificity of 100%. The results indicate that the mutations in HPs, even if they come from a very small tumor, can be detected in the stool DNA. It also proves that the technique of stool-based DNA detection for colorectal tumors is very sensitive.

Considering the small sample numbers and selected populations of symptomatic patients in our study, large investigations of fecal DNA analysis using these genes in asymptomatic populations are needed. In addition, recent investigations have shown that methylation of the hMLH1 is very common in HPs with MSI[5]. Thus, the combinations of genetic markers with epigenetic methylated genes will probably increase the sensitivity of the colorectal polyps detection rate.

In summary, our data indicate that mutations of BRAF, K-ras and BAT26 genes in adenomas and HPs are frequent and can be detected in corresponding stool samples. They

can be used as stool based genetic markers for detection of colon polyps.

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Synergistic effect of a novel oxymatrine-baicalin combination against hepatitis B virus replication, α smooth muscle actin expression and type I collagen synthesis *in vitro*

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Abstract

AIM: To study the effect of oxymatrine-baicalin combination (OB) against HBV replication in 2.2.15 cells and α smooth muscle actin (α SMA) expression, type I, collagen synthesis in HSC-T6 cells.

METHODS: The 2.2.15 cells and HSC-T6 cells were cultured and treated respectively. HBsAg and HBeAg in the culture supernatants were detected by ELISA and HBV DNA levels were determined by fluorescence quantitative PCR. Total RNA was extracted from HSC-T6 cells and reverse transcribed into cDNA. The cDNAs were amplified by PCR and the quantities were expressed in proportion to β actin. The total cellular proteins extracted from HSC-T6 cells were separated by electrophoresis. Resolved proteins were electrophoretically transferred to nitrocellulose membrane. Protein bands were revealed and the quantities were corrected by β actin.

RESULTS: In the 2.2.15 cell culture system, the inhibitory rate against secretion of HBsAg and HBeAg in the OB group was significantly stronger than that in the oxymatrine group (HBsAg, $P = 0.043$; HBeAg, $P = 0.026$; respectively); HBV DNA level in the OB group was significantly lower than that in the oxymatrine group ($P = 0.041$). In HSC-T6 cells the mRNA and protein expression levels of α SMA in the OB group were significantly lower as compared with those in the oxymatrine group (mRNA, $P = 0.013$; protein, $P = 0.042$; respectively); The mRNA and protein expression levels of type I collagen in the OB group were significantly lower as compared with those in the oxymatrine group (mRNA, $P < 0.01$; protein, $P < 0.01$; respectively).

CONCLUSION: OB combination has a better effect against HBV replication in 2.2.15 cells and is more effective against α SMA expression and type I collagen synthesis in HSC-T6 cells than oxymatrine *in vitro*.

Key words: 2.2.15 cells; HSC-T6 cells; Oxymatrine; Baicalin; Hepatitis B virus; α smooth muscle actin; Type I collagen

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INTRODUCTION

The human hepatitis B virus (HBV) belongs to the family of small DNA hepadnaviruses. HBV infection is a major cause of chronic hepatitis, hepatic fibrosis, liver cirrhosis, and hepatocellular carcinoma and results in one million deaths annually[1]. In China there are about thirty million patients suffering from chronic hepatitis B. Hepatic fibrosis is a kind of compensating and healing response to liver injuries. And hepatic fibrogenesis has been known to be a gradual and dynamic process associated with the continuous deposition and resorption of connective tissues and collagens[2,3]. During the hepatic fibrogenesis process, hepatic stellate cells (HSC, formerly termed as lipocytes, Ito cells or fat-storing cells) play a central role based on their ability to undergo activation following liver injury of any cause. HSC has been recognized to be responsible for most of the excess extracellular matrix (ECM) observed in chronic hepatic fibrosis[4].

For a few decades, interferon α (IFN α) has been the only approved therapy for chronic HBV infection around the world. But its efficacy is not satisfactory and associated with some adverse reactions such as influenza-like syndrome, leukocyte and platelet decrease[5]. Recently, lamivudine has been the first nucleotide analog approved for treating chronic HBV infection in many regions of the world; but its efficacy is just similar to IFN α and prolonged administration is associated with drug

resistance and virus variation, which could lead to severe consequences including liver failure[6] and even death[7]. Up to now, the treatment of HBV is still a difficult problem. Therefore, it is necessary to develop novel drugs and treatment methods.

Oxymatrine is a kind of alkaloid extraction derived from a Chinese herb *Sophora flavescens* Ait[2,3]. It has been widely used for treating viral hepatitis B and C and hepatic fibrosis in recent years in China[8,9]. But just like other anti-HBV drugs, how to improve its efficacy against HBV and hepatic fibrosis is still an urgent challenge in clinical practice. On the basis of our previous findings[10], in the present study, the 2.2.15 cells[11], the hepatoblastoma cell line HepG2 transfected with cloned hepatitis B virus DNA, and the rat HSC-T6 cells[12], an immortalized rat hepatic stellate cell line, were cultured respectively, and the effects of a novel oxymatrine-baicalin combination[10] (OB) against HBV replication and α smooth muscle actin (α SMA) expression, type I collagen synthesis *in vitro* were evaluated.

MATERIALS AND METHODS

Drugs, reagents and instruments

Oxymatrine, and OB combination were prepared and provided by Shanghai Kairuisi Biotech Co. Ltd. according to our applied China National Invention Patent[13]. Dulbecco's modified Eagle's medium (DMEM) and modified Eagle's medium (MEM) culture media were the products of Gibco BRL. Fetal bovine sera (FBS) were purchased from Hyclone (Logan, Utah, USA). G418 was purchased from Shanghai Jiebeisi Gene-Tech Co. Ltd. *L*-glutamine and EDTA were supplied by Shanghai Shisheng Biotech Co. Ltd. The ELISA kits for HBsAg (hepatitis B surface antigen) and HBeAg (hepatitis B e antigen) kits were ordered from Huamei Biotech Co. Ltd. The penicillin and streptomycin were the products of Shanghai Xianfeng Pharmacological Co.

The HBV DNA (deoxyribonucleic acid) PCR (polymerase chain reaction)-fluorescence quantitative diagnostic kit was purchased from Shanghai Kehua Bio-engineering Co. Ltd. Trizol reagent was the product of Invitrogen. DNA marker was purchased from Tiangen Co. The cDNA synthesis kit and PCR master mix kit were purchased from Fermentas Co. The specific primers were synthesized by Shanghai Shenggong Co. The rabbit anti-rat type I collagen polyclonal antibody was the product of Merck. The mouse anti-rat α SMA mAb and anti-rat β actin mAb were purchased from Sigma (St. Louis, MO). The DC protein quantification kit was the product of Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). Enhanced chemiluminescence reagents were purchased from Pirce. The other reagents routinely used in our laboratory were all of analytical grade.

Cell culture flasks and plates were the products of Corning Inc (Corning, NY, USA). CO_2 culture hood and Healforce hood were the products of Heraeus Co. Labsystems Multiskan MS Microplate Reader was made in Finland. XDS-1B invert phase-contrast microscope was purchased from Chongqin Guangdian Co. The LightCycler fluorescence PCR system was the product of Roche Co. The Rotor-Gene RG-3000 PCR machine was the product of Gene Co. The Furi FR-980 image analysis system was provided by Shanghai Furi Co. Western blot instrument was produced by Bio-Rad.

Cell culture and treatment

The 2.2.15 cell line was purchased from the Department of Microbiology, Tianjin Medical University, China. The 2.2.15 cells were cultured in complete MEM culture media (supplemented with 100 mL/L FBS, 3.8 g/L *L*-glutamine, 0.38 g/L G418, 50 ku/L penicillin and streptomycin, pH 7.0) at 37℃ in 50 mL/L CO_2, 950 mL/L air. When cells were in the logarithmic growth phase, they were trypsinized and seeded. Experiments were performed when cells reached 80% confluence. The cells were supplemented with oxymatrine or OB combination and incubated for an additional 4 d. The working concentration of oxymatrine was 1 g/L and the OB combination contained 1 g/L oxymatrine[10] and a specific concentration of baicalin[13] according to our previous findings. At the above concentrations these drugs have no cytotoxic effect to cells by MTT colorimetric assay[10]. Then the cell culture medium was collected according to the experimental protocol. All experiments were performed in duplicate or triplicate samples.

HSC-T6 cells were kindly provided by Professor Scott Friedman (Liver Center Laboratory, San Francisco General Hospital, USA) and has been stored and passaged routinely in our institute. HSC-T6 cells were seeded in DMEM with 100 mL/L FBS at 37℃, in 50 mL/L CO_2, 950 mL/L air. When cells were in the logarithmic growth phase, they were trypsinized and seeded into 60 mm culture plates. Experiments were performed when cells reached 80% confluence. Each plate was supplemented with 2 mL culture media with oxymatrine or OB combination and incubated for an additional 24 h. Then the cells were harvested according to the experimental protocol. All experiments were performed in duplicate or triplicate samples.

Measurement of HBV antigens secretion in supernatants of 2.2.15 cells by ELISA

The 2.2.15 cells were incubated at a density of 1×10^9/L in 1 L MEM medium containing 100 mL/L FBS. After 24 h incubation, the 2.2.15 cells were treated with oxymatrine or OB combination respectively. Cells were cultured in the presence of drugs for 4 d. Then the supernatants were collected and stored at -20℃ until measurement. The HBsAg and HBeAg were detected simultaneously by ELISA kits according to the manufacturer's instruction, and the inhibitory rates for HBsAg and HBeAg were calculated respectively.

Measurement of HBV DNA replication in supernatants of 2.2.15 cells by fluorescence quantitative PCR

The HBV DNA level in the supernatants post-drug treatment was determined by the HBV DNA PCR-quantitative diagnostic kit using the Roche LightCycler system according to the manufacturer's protocol. One hundred microliter culture medium was added to 100 μL sample reagent A, and then centrifuged at 13000 r/min

for 10 min. The supernatants were discarded and 25 μL sample reagent B was added. After centrifugation for a few seconds, the mixture was heated at 100℃ for 10 min. Then 2 μL of the supernatants after centrifugation at 13000 r/min for 10 min was mixed with 18 μL PCR reaction reagent, and analyzed at Channel F1 by the LightCycler system.

Determination of α SMA and type I collagen mRNA levels by semi-quantitative RT-PCR

Total RNA was extracted from HSC-T6 cells by Trizol reagent following the protocols provided by the manufacturer. The integrity of total RNA was confirmed by the denaturing formaldehyde agarose gel electrophoresis. The quantity and purity of RNA were detected by determining absorbance at 260/280 nm using a spectrophotometer. Total RNA was reverse transcribed into complementary DNA (cDNA) using the cDNA synthesis kit. The reverse-transcription (RT) reaction mixtures were amplified by semi-quantitative PCR using specific primers for the target genes and β actin. The primers used are as follows: Type I collagen, forward: 5'-TAC AGC ACG CTT GTG GAT G-3', reverse: 5'-TTG AGT TTG GGT TGT TGG TC-3', target fragment length 259 bp; α SMA, forward: 5'-CCG ACC GAA TGC AGA AGG-3', reverse: 5'-ACA GAG TAT TTG CGC TCC GGA-3', target fragment length 88 bp; β actin, forward: 5'-TGA CGA GGC CCA GAG CAA GA-3', reverse: 5'-ATG GGC ACA GTG TGG GTG AC-3', target fragment length 330 bp. The 20 μL reaction mixture consisted of the corresponding primers, 1 U of Taq polymerase, 50 μmol/L of each of the four dNTP, 1 × PCR buffer supplemented with 2.5 mmol/L $MgCl_2$. After an initial melting time of 5 min at 94℃, the mixtures were subjected to 35 PCR cycles, consisting of denaturation for 1 min at 94℃, primer annealing for 1 min at 55℃, and primer extension for 1 min at 72℃, with a final extension time of 10 min at 72℃. Quantification of the final products was performed by electrophoresis in 30 g/L agarose gel using the Furi FR-980 image analysis system. The number of mRNA molecules was expressed in proportion to the number of internal control β actin in the same sample as described before[14].

Determination of α SMA and type I collagen protein levels by Western blot

HSC-T6 cell lysates were prepared from 1×10^7 cells by dissolving cell pellets in 100-200 μL lysis buffer (150 mmol/L NaCl, 10 g/L tergitol NP-40, 5 g/L sodium deoxycholate, 0.1 g/L sodium dodecyl sulfate, 50 mmol/L Tris, and a protease inhibitor cocktail, pH7.5). Lysates were centrifuged at 4℃, 12000 r/min for 15 min, then the supernatants were collected and stored at -70℃ until detection. Protein content was analyzed by DC protein assay and at 690 nm wavelength. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.31 mL 2 mol/L Tris buffer (pH6.8), 20 g/L SDS, 100 mL/L glycerol, 2.4 g/L DTT, 50 g/L β-mercaptoethanol, 0.02 g/L bromophenol blue) was added to the lysates. After mixing the lysates were heated to 100℃ for 5 min, and 50 μg sample protein was loaded into each well of 100 g/L SDS-PAGE gel. Resolved proteins were electrophoretically transferred to nitrocellulose membrane and blocked with 50 mL/L non-fatty milk. Then the primary antibodies (mouse anti-rat β actin antibody, 1:5000 dilution; mouse anti-rat α SMA antibody, 1:5000 dilution, rabbit anti-rat type I collagen antibody, 1:200 dilutions) in 50 mL/L non-fatty TTBS solution (1.21 g/L Tris, 9 g/L NaCl, 1 g/L Tween-20, pH7.5) were added respectively. After incubation at 4℃ overnight the blots were washed, then membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies for 1 h. Protein bands were revealed by the ECL kit according to the manufacturer's protocol and the Furi FR-980 image analysis system was used for quantitative analysis of the blots. β actin protein was used as the internal control as described before[15].

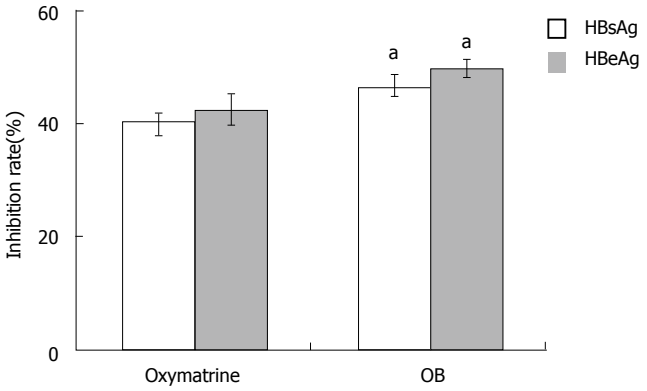

Figure 1 The inhibition rate of OB on HBsAg and HBeAg secretion in the 2.2.15 cell culture system. $n = 3$, mean ± SD. $^aP < 0.05$ *vs* oxymatrine.

Statistical analysis

All results were expressed as mean ± SD. Comparisons were analyzed by one-way ANOVA using the SPSS 10.0 statistical package. Differences were considered statistically significant if the $P < 0.05$.

RESULTS

Effect of OB on HBV antigen secretion in 2.2.15 cells

After incubation for 4 d, HBsAg and HBeAg secretion in the culture medium was determined by ELISA. As shown in Figure 1, both OB and oxymatrine had remarkable inhibitory effects on secretion of HBsAg and HBeAg in the 2.2.15 cells, the inhibitory rate in the OB group was significantly stronger than that in oxymatrine group (HBsAg, $P = 0.043$; HBeAg, $P = 0.026$; respectively). And the inhibitory rate on HBeAg in both groups exceeded that of HBsAg.

Effect of OB on HBV DNA replication in 2.2.15 cells

After incubation for four days, the levels of HBV DNA in the culture medium were determined by fluorescence quantitative PCR. As shown in Figure 2, both OB and oxymatrine reduced the HBV DNA level in the supernatant of the 2.2.15 cells, and the HBV DNA level in the OB group was significantly lower than that of the oxymatrine group ($P = 0.041$), indicating that OB inhibited

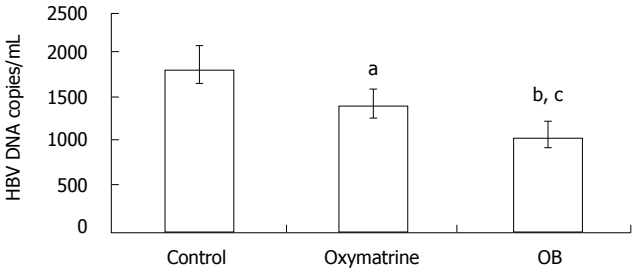

Figure 2 The effect of OB on the HBV DNA level in the 2.2.15 culture system. $n = 3$, mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control, $^{c}P < 0.05$ *vs* oxymatrine.

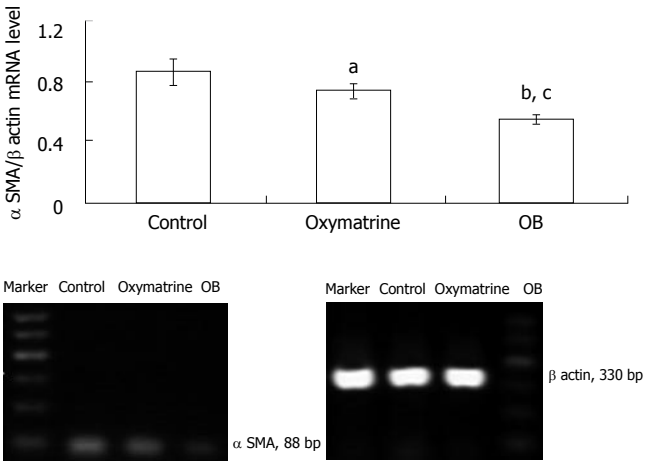

Figure 3 Effect of OB treatment on the α SMA mRNA level in HSC-T6 cells. The α SMA mRNA level was determined by semi quantitative RT-PCR analysis and corrected by β actin mRNA level. $n = 3$, mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control, $^{c}P < 0.05$ *vs* oxymatrine.

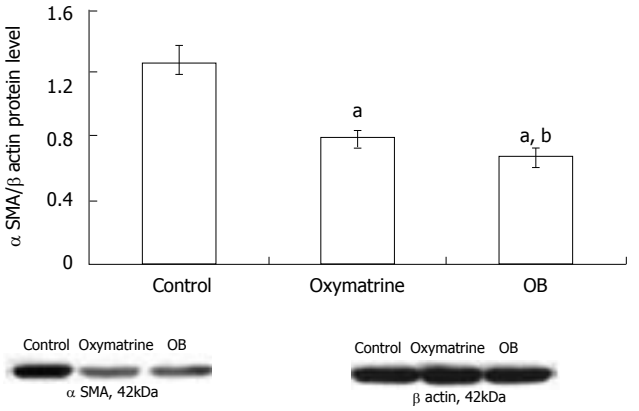

Figure 4 Effect of OB treatment on the α SMA protein level in HSC-T6 cells. The α SMA protein level was determined by Western blot analysis and corrected by β actin protein level. $n = 3$, mean ± SD. $^{b}P < 0.01$ *vs* control, $^{a}P < 0.05$ *vs* oxymatrine.

HBV DNA replication more effectively than oxymatrine did.

Effect of OB on α SMA expression in HSC-T6 cells

The α SMA mRNA and protein expression levels in HSC-T6 cells were determined by semi-quantitative PCR and Western blot analysis respectively. The β actin mRNA and protein were chosen as internal controls. The results

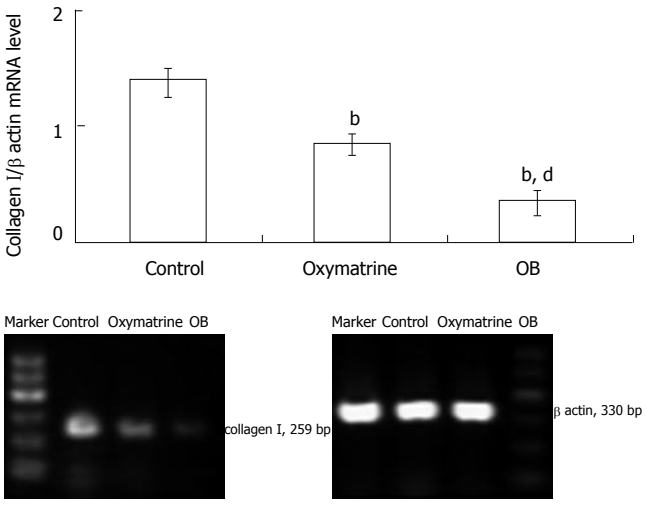

Figure 5 Effect of OB treatment on the Collagen I mRNA level in HSC-T6 cells. The collagen mRNA level was determined by semi quantitative RT-PCR analysis and corrected by the β actin mRNA level. mean ± SD. $^{b}P < 0.01$ *vs* control, $^{d}P < 0.01$ *vs* oxymatrine.

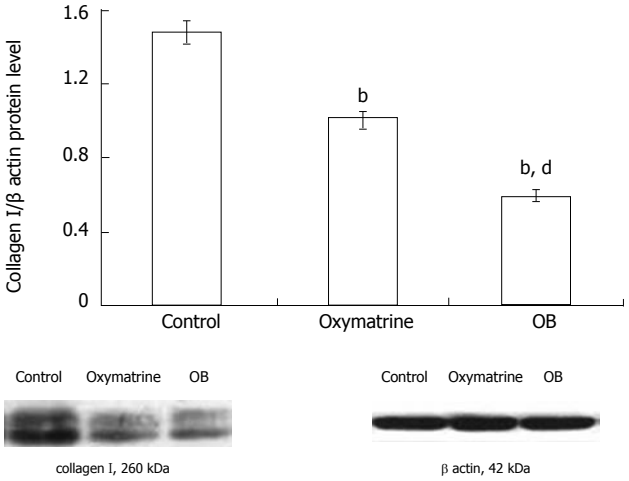

Figure 6 Effect of OB treatment on the collagen I protein level in HSC-T6 cells. The collagen protein level was determined by Western blot analysis and corrected by the β actin protein level. $n = 3$, mean ± SD. $^{b}P < 0.01$ *vs* control, $^{d}P < 0.01$ *vs* oxymatrine.

showed that the mRNA and protein expression levels of α SMA in both the oxymatrine and OB groups were significantly reduced compared with the control group (mRNA, $P = 0.033$ and $P = 0.001$ *vs* the control; protein, $P < 0.01$ and $P < 0.01$ *vs* the control; respectively). As shown in Figures 3 and 4, the mRNA and protein expression levels of α SMA in the OB group were significantly lower as compared with those in the oxymatrine group, suggesting that OB reduced the HSC-T6 activation more effectively than oxymatrine (mRNA, $P = 0.013$; protein $P = 0.042$; respectively).

Effect of OB on type I *collagen synthesis in HSC-T6 cells*

The type I collagen mRNA and protein expression levels were also determined. As shown in Figures 5 and 6, the mRNA and protein expression levels in both the oxymatrine and OB groups were significantly reduced compared with the control group (mRNA, $P < 0.01$ and P

< 0.01; protein, $P < 0.01$ and $P < 0.01$; respectively). The results showed that the mRNA and protein expression levels in the OB group were significantly lower as compared with those in the oxymatrine group, suggesting that the OB combination reduced the synthesis of type I collagen in HSC-T6 cells more effectively than oxymatrine not only at mRNA level but also at the protein level (mRNA, $P < 0.01$; protein, $P < 0.01$; respectively).

DISCUSSION

The World Health Organization places HBV infection among the top 10 causes of death worldwide. It is estimated that there are over 400 million carriers of HBV. At least 20% to 30% of HBsAg carriers will die of complications of chronic liver disease, including cirrhosis and liver cancer. The serious consequences of end-stage liver disease and liver cancer occur in 30% of chronic carriers[1,5,16]. Investigation of the expression and replication of the HBV genome has been hampered by the lack of an *in vitro* tissue culture system until the 2.2.15 cell line was established[11]. The medium of these cells contains not only all of the particles present in the serum of infected individuals but also a number of replicative intermediates that probably represent recircularized, ccc, and single-stranded HBV DNA. Various parameters of the replicative cycle can be quantitated in the 2.2.15 culture system, for example, the secretion of HBsAg or HBeAg and the amount of episomal HBV DNA. At present, the 2.2.15 cell line is still the main *in vitro* model used for evaluating the anti-HBV effect of drugs[10].

Because HBV infection plays a key role in the development of decompensated cirrhosis and hepatocellular carcinoma, HBV patients with serum aminotransferase twice the upper limit of reference value and HBV DNA positive in the blood were advised to receive antiviral agents treatment[5,8]. Oxymatrine has been used widely in the treatment of chronic liver disease including hepatitis B, hepatitis C and hepatic fibrosis[2,3,7,8]. As for etiological treatment, oxymatrine could effectively treat chronic viral hepatitis and promote the serum markers of HBV in chronic hepatitis B patients to convert to negative and reduce serum level of ALT[8,9]. But its efficacy is similar to IFN α, therefore, it is urgent to improve the treatment efficacy of oxymatrine. The present study showed that the inhibitory rate of OB combination against HBV antigens secretion in the supernatant of the 2.2.15 culture system was significantly stronger than that of oxymatrine. In order to elucidate the influence of OB combination on HBV replication, HBV DNA was determined in the supernatants also and the results indicated that OB combination could suppress virus replication and the effect was superior to oxymatrine treatment alone. The pattern of OB combination against HBV is consistent with those reports on oxymatrine elsewhere[16,17].

The objective of anti-HBV therapy is not only to eradicate the virus but also to prevent the development of hepatic fibrosis. Hepatic fibrosis, a precursor of cirrhosis, is a consequence of severe liver damage that occurs in many patients with chronic liver disease, and involves the abnormal accumulation of ECM[2,3,9,14]. Liver fibrosis represents a major healthcare burden worldwide. During hepatic injury, HSCs become active and undergo profound phenotypic changes[4,18]. HSCs are non-parenchyma cells, located perisinusoidally in the space of Disse. The expression of α SMA is the most important feature of HSC activation. HSC is the primary source of excessive production of ECM. The collagens are the major ECM component of normal and fibrotic livers. In the normal liver the amount of type III collagen is greater than type I collagen, but type I collagen is particularly produced predominantly during fibrogenesis. While the primary HSC cultures are a useful tool for studying hepatic fibrogenesis, their isolation is extremely time-consuming, the yields are modest, and there is considerable preparation-to-preparation variability. The rat HSC-T6 cell line[12] is constructed through transfecting SV40 into rat HSC and its phenotype is activated HSC. HSC-T6 has the stable phenotype and biochemical characters of activated HSC, expressing myogenic and neural crest cytoskeletal filaments, and the cell line has been a useful tool for studying hepatic fibrogenesis and it is also a reliable cell model for investigating antifibrotic drugs[19].

In this study the RT-PCR and Western blot analysis results showed that OB combination had a stronger inhibitory effect against the activation of HSC, and this was proven by the significantly lower levels of α SMA mRNA and protein in the OB group than those in the oxymatrine group. Type I collagen is produced predominantly during hepatic fibrogenesis[4,18]. Inhibiting the secretion of type I collagen not only reduced the ECM component, but also decreased further activation of HSC[19]. Our results showed that the OB combination could not only inhibited the synthesis of type I collagen at the transcription level but also at the translation level, and these inhibitory effects of the OB combination were superior to those of oxymatrine.

In addition to the anti-HBV effect[8,16], it was reported that oxymatrine has an effect against liver fibrosis *in vitro*[15] and *in vivo*[2,3,17,21], protects animals from fulminant hepatitis[22] or inflammation[23]. Baicalin, a flavonoid isolated from the root of *Scutellaria baicalensis* Georgi[10], has been demonstrated to have multiple biological functions, such as anti-HBV[9,24], inhibiting HIV infection[25], inhibiting SARS coronavirus[26]. Romero[24] confirmed that baicalin has a moderate ability to reduce HBV production and has no toxic effect on host cells, and this effect against HBV was confirmed by our findings[10] also. Moreover, it was reported that baicalin[25] at the noncytotoxic concentrations inhibited both T cell tropic (X4) and monocyte tropic (R5) HIV-1 Env protein mediated fusion with cells expressing CD4/CXCR4 or CD4/CCR5, and the presence of baicalin at the initial stage of HIV-1 viral adsorption blocked the replication of HIV-1 , resulting in an early definitive DNA replication cessation. Chen[27] found that baicalin had antiviral activity against 10 clinical isolates of SARS coronavirus by neutralization tests, which was confirmed by plaque reduction assays. Jang[28] reported that baicalin had a protective effect against acetaminophen-induced hepatotoxicity in mice and showed that the effects might be due to a block of the bioactivity of acetaminophen by inhibiting the cytochrome P450 2E1 expression. As an antioxidant flavonoid *in vitro*

baicalin has a strong antioxidant activity toward reactive oxygen species (ROS), including hydroxyl radical (OH·), superoxide anions (O_2·) and peroxynitrite ($ONOO_2$), and inhibits lipid peroxidation, promoting the repair of DNA single strand breakage caused by H_2O_2 in cultured NIH3T3 fibroblasts[29]. It has long been accepted that Sho-saiko-to functions as a potent anti-hepatic fibrosis agent, and Japanese investigators confirmed that the active components of Sho-saiko-to are baicalin and baicalein of flavonoids, and the chemical structures of baicalin and baicalein are very similar to silybinin, which shows anti-fibrogenic activities[30,31].

The mechanism of hepatic fibrosis is too complicated for a single drug to resolve. A drug or drug compounds with multi-effect-pathways and multi-effect-targets may have better efficacy than a single drug alone, and could change the current predicament in the therapy of hepatic fibrosis[19]. Continued progress is essential in order to identify the determinants and dynamics of fibrosis reversibility, to discover additional targets for anti-fibrotic therapy, and to develop customized multi-drug regimens[32]. Taken together, the results from 2.2.15 cells show that the inhibitory effect of OB combination on HBV antigen secretion and HBV DNA replication is better than that of oxymatrine alone; furthermore, the results from HSC-T6 also show that the suppressing effect of OB combination on α SMA and type I collagen expression is more effective than that of oxymatrine. This study indicates that the addition of baicalin to oxymatrine can strengthen the treatment effect of oxymatrine *in vitro*. However, this finding needs to be verified in *in vivo* studies.

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BASIC RESEARCH

Signaling pathways involved in the inhibition of epidermal growth factor receptor by erlotinib in hepatocellular cancer

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Abstract

AIM: To examine the underlying mechanisms of erlotinib-induced growth inhibition in hepatocellular carcinoma (HCC).

METHODS: Erlotinib-induced alterations in gene expression were evaluated using cDNA array technology; changes in protein expression and/or protein activation due to erlotinib treatment as well as IGF-1-induced EGFR transactivation were investigated using Western blotting.

RESULTS: Erlotinib treatment inhibited the mitogen activated protein (MAP)-kinase pathway and signal transducer of activation and transcription (STAT)-mediated signaling which led to an altered expression of apoptosis and cell cycle regulating genes as demonstrated by cDNA array technology. Overexpression of proapoptotic factors like caspases and gadds associated with a down-regulation of antiapoptotic factors like Bcl-2, Bcl-X_L or jun D accounted for erlotinib's potency to induce apoptosis. Downregulation of cell cycle regulators promoting the G_1/S-transition and overexpression of cyclin-dependent kinase inhibitors and gadds contributed to the induction of a G_1/G_0-arrest in response to erlotinib. Furthermore, we displayed the transactivation of EGFR-mediated signaling by the IGF-1-receptor and showed erlotinib's inhibitory effects on the receptor-receptor cross talk.

CONCLUSION: Our study sheds light on the understanding of the mechanisms of action of EGFR-TK-inhibition in HCC-cells and thus might facilitate the design of combination therapies that act additively or synergistically. Moreover, our data on the pathways responding to erlotinib treatment could be helpful in predicting the responsiveness of tumors to EGFR-TKIs in the future.

Key words: Epidermal growth factor receptor; Insulin-like growth factor receptor; Tarceva™; Signal transducer of activation and transcription; Extracellular regulated kinase; Gene expression

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world and is estimated to cause half a million deaths annually. The incidence of HCC is dramatically increasing in the USA, Europe and Asia, due to high prevalence of chronic hepatitis B and hepatitis C virus infections, alcohol disease, diabetes and obesity[1]. Unfortunately, the majority of patients suffer from advanced disease at presentation. Therefore curative local ablation, surgical resection of HCC, or liver transplantation can be achieved only in a minority of patients. Local tumor destruction, chemoembolization or systemic chemotherapy are the treatment options of advanced HCC. However, overall survival is poor. Apart from chemoembolization, which improves survival in well-selected patients with unresectable HCC, palliative treatment options do not appear to greatly improve overall survival[2]. Therefore, innovative treatment approaches are urgently needed. Recently, evidence has been accumulated that the epidermal growth factor receptor (EGFR) is a promising target for cancer therapy. A great variety of tumors show abnormal, enhanced and/or constitutive expression of EGFR. Several reports indicate that EGFRs are expressed frequently in human HCC, most likely contributing to the aggressive growth characteristics of these tumors[3,4]. Especially in poorly differentiated HCCs, EGFR overexpression has been demonstrated to be a negative prognostic factor, since it positively correlated with early

tumor recurrence and the occurrence of extrahepatic metastasis[3,5]. Hence, the EGFR is a promising target for innovative treatment strategies in HCC.

The EGFR is a member of a family of four closely related receptors: EGFR (ErbB-1), HER-2/*neu* (ErbB-), HER-3 (ErbB-3), and HER-4 (ErbB-4). Upon ligand binding the EGFR becomes activated by dimerization which leads to subsequent activation of EGFR tyrosine kinase (TK) activity, initiating receptor-mediated signal transduction, cell mitogenesis and cell transformation[6]. The EGFR downstream intracellular signal transduction pathways include components of Ras/mitogen-activated protein kinase (MAPK), phosphatidyl inositol 3-kinase, signal transducer and activator of transcription (STAT), downstream protein kinase C and phospholipase D pathways[7]. The Ras/MAPK cascade is supposed to be one of the major signaling routes of the EGFR system[8].

Erlotinib [N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine] is a novel orally available low-molecular-weight quinazolinamine that acts as a potent and reversible inhibitor of EGFR-TK activity. The mechanism of action of erlotinib is competitive inhibition of the binding of ATP to the TK domain of the receptor, resulting in inhibition of EGFR autophosphorylation[9]. Single agent activity was observed in pretreated patients with non-small-cell lung cancer(NSCLC), head and neck carcinoma and ovarian cancer[10]. Recently, the results of the BR.21 phase III trial showed a significant 42.5% improvement in median survival compared to placebo in patients with advanced NSCLC[11] and the US Food and Drug Administration (FDA) has approved erlotinib for this indication in November 2004.

In a previous study we have shown that EGFR-TK-inhibition by erlotinib potently suppresses the growth of human EGFR-expressing HCC cells by inducing both apoptosis and cell cycle arrest at the G_1/S-transition[12].

The objective of the current study was to examine the underlying mechanisms of erlotinib-induced growth inhibition in HCC cells. For this purpose we studied the effects of erlotinib on downstream signaling molecules of the EGFR. We used cDNA array technology to investigate the EGFR-TKI-induced modulation of apoptosis- and cell cycle-related genes and Western blot analysis to evaluate changes in the activation of the mitogenic MAP-kinase- and Jak-STAT-pathways as well as changes in the expression of cell-cycle regulating and antiapoptotic proteins. Additionally, we investigated the influence of IGF-1R-activation on EGFR-mediated signaling and erlotinib's effects on the IGF-1R/EGFR-network.

MATERIALS AND METHODS

Materials

The highly differentiated human hepatocellular carcinoma cell line Huh-7 and the well differentiated hepatoblastoma cell line HepG2 were cultured in RPMI 1640 medium containing 100 mL/L fetal bovine serum and 100 kU/L penicillin and 100 mg/mL streptomycin. Erlotinib hydrochloride was a kind gift from Roche (Penzberg, Germany), cell culture material was from Biochrom (Berlin, Germany); all other chemicals were from Sigma (München, Germany), if not stated otherwise. Stock solutions were prepared in DMSO and stored at -20℃ and were diluted to the final concentration in fresh media before each experiment. In all experiments, the final DMSO concentration did not exceed 5 g/L, thus not affecting cell growth. To evaluate the effects of erlotinib, cells were incubated with either control medium or medium containing rising concentrations of erlotinib.

Drug combination studies

To check for possible additive or synergistic effects, combination treatment of erlotinib plus AG1024 (Calbiochem, Bad Soden, Germany) was studied. The 5 μmol/L or 10 μmol/L of the tyrphostine AG1024 was combined with 10 μmol/L erlotinib (e.g. approximately its IC_{50} value). The antineoplastic activities of the combinations were compared to those of each drug alone. For all experiments cell number was evaluated by crystal violet staining as described[12]. In brief, cells in 96-well plates were fixed with 10 g/L glutaraldehyde, then cells were stained with 1 g/L crystal violet in PBS. The unbound dye was removed by washing with water. Bound crystal violet was solubilized with 2 g/L Triton-X-100 in PBS. Light extinction which increases linearly with the cell number was analyzed at 570 nm using an ELISA-reader.

Western blot analysis

Western blotting was performed as described[13]. Blots were blocked in 2.5% BSA and then incubated at 4℃ overnight with the following antibodies: ERK1/2 (1:500), p-ERK1/2 (1:500), cyclin D1 (1:100), Bcl-X_L (1:200), STAT1 (1:1000), STAT3 (1:1000), STAT5 (1:1000), β-IGF-1R (1:1000), $p21^{Waf1/CIP1}$ (1:200; all from Santa Cruz Biotechnology, CA), $p27^{KIP1}$ (1:2500; Becton-Dickinson, Heidelberg, Germany), p-EGFR, p-STAT1(TYR701), p-STAT3(TYR705), p-STAT5 (TYR694) (all 1:500 and all from Cell Signaling, MA) and p-IGF-1R (1:1500; Biomol, Hamburg, Germany). β-actin (1:5000; Sigma, Deisenhofen, Germany) served as loading control. One representative out of three independent experiments was shown for each Western blot.

RNA extraction and poly(A)$^+$ mRNA preparation

Total RNA was extracted from cultured HepG2 cells with RNAClean according to the munufacturer's recommendations (Hybaid, London, UK). Polyadenylated (poly(a)$^+$) mRNAs were enriched using magnetic Dynabeads according to the instructions of the supplier (Dynal, Oslo, N). The quality of poly(A)$^+$ and total RNA was assessed by agarose gel electrophoresis.

cDNA array

HepG2 cells were treated with 10 μmol/L erlotinib for 48 h to determine erlotinib-induced differential gene expression. Untreated cells served as controls. We used the Atlas Human Apoptosis cDNA array with 205 human cDNAs spotted in duplicate on a nylon membrane (Clontech, Palo Alto, CA) as previously described[14]. A complete list of the cDNAs and controls as well as their accession numbers is available on the web (http://www.clontech.com/clontech/atlas/genelists/7743-1_HuApop.pdf). The hybridization signals were

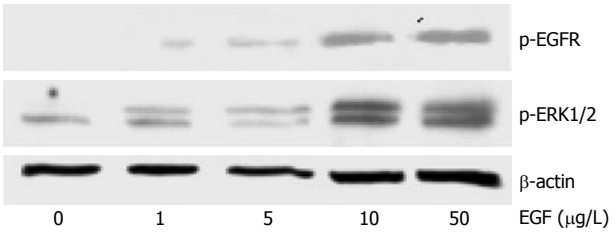

Figure 1 EGF-induced EGFR- and ERK1/2-activation in HCC cells.

photometrically evaluated using TINA software (Raytest Isotopenmessgeräte, Straubenhardt, D). Altered expression of a respective gene is given as a fold increase or decrease compared to the signal of the control. Data analysis was performed as described[14].

Statistical analysis

The means of three independent experiments of the cDNA expression arrays and the drug combination studies ± SD are shown. Significance between AG1024 treated samples and AG1024 plus erlotinib treated samples was calculated by Student's two sided *t*-test. $P < 0.05$ was regarded as significant.

RESULTS

EGF-induced EGFR- and ERK1/2-activation

To shed light on the signaling pathways modulated by EGFR-TK inhibition in HCC cells, we investigated the phosphorylation of ERK1/2 known to be involved in EGFR-mediated mitogenic and antiapoptotic signaling. In order to demonstrate the influence of the epidermal growth factor (EGF) on the activation of the EGFR and ERK1/2, serum-starved HepG2 cells were incubated for 15 min with increasing concentrations of EGF (1-50 μg/L). Serum-starved cells were chosen to exclude the influence of growth factors contained in the fetal calf serum (FCS) of the cell medium. Western blotting of whole cell lysates revealed a dose-dependent increase of activated EGFR and ERK1/2 in response to EGF incubation (Figure 1).

Inhibitory action of erlotinib on EGF-induced EGFR- and ERK1/2 activation

EGF-induced activation of the EGFR and the mitogenic ERK1/2 was blocked by pretreating the cells with erlotinib. HepG2 cells were incubated for 30 min with escalating concentrations of erlotinib (0.1, 1, 10 μmol/L) and subsequently stimulated with EGF (10 μg/L). Again, activation of EGFR and ERK1/2 was determined by Western blotting. Erlotinib-untreated cells (control) displayed a pronounced activation of EGFR and ERK1/2 due to EGF-stimulation. Low concentrations of erlotinib (0.1 μmol/L) completely blocked EGFR-phosphorylation and ERK1/2-activation decreased dose-dependently (Figure 2A).

To mimic *in vivo* conditions, we finally examined the influence of erlotinib (10 μmol/L) on the activation of ERK1/2 in HepG2 cells stimulated by growth factors contained in the medium's FCS. We revealed a time-dependent decrease in phosphorylation of ERK1/2 (Figure 2B) in HCC cells due to erlotinib treatment (up to 72 h).

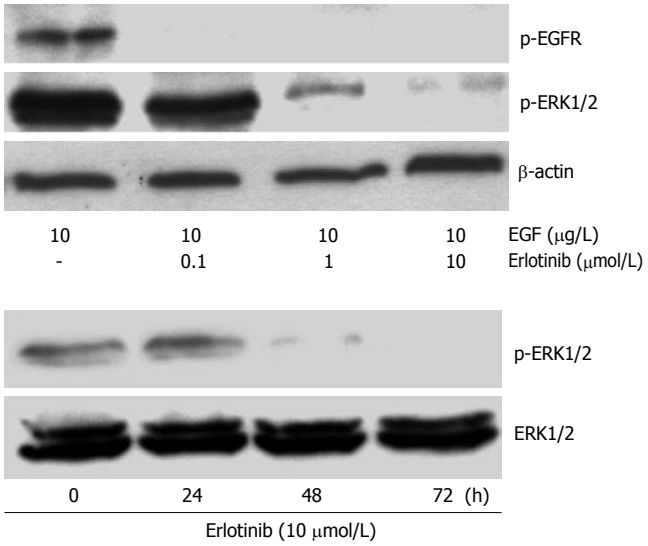

Figure 2 Erlotinib inhibited EGFR (**A**) and ERK1/2 (**B**) activation in HCC cells.

STAT expression and EGF-induced activation

EGFR activation is supposed to result in autophosphorylation of Janus-kinases (Jaks) with subsequent activation of mitogenic STATs but has not been investigated in HCC so far. Western blot analyses revealed the expression of STATs 1, 3 and 5 in Huh-7 cells (Figures 3A-C). Moreover, EGF induced STAT activation could be shown for STATs 1 and 3 (Figures 3A and B) but not in the case of STAT5 (Figure 3C). Serum-starved Huh-7 cells were stimulated with EGF (10 or 50 μg/L, 15 min) and whole cell lysates were used for determinations.

Inhibitory effect of erlotinib on STAT-activation

EGF-induced STAT activation was blocked by pretreating Huh-7 cells with erlotinib. Erlotinib-untreated cells (control) displayed a pronounced activation of STATs 1 and 3 in response to EGF-incubation (10 μg/L, 15 min). Erlotinib pretreatment (0.1, 1, 10 μmol/L; 30 min) dose-dependently inhibited the phosphorylation of STATs 1 and 3, whereas the total quantity of STAT 1 and 3 remained unchanged (Figure 4; +: addition of the respective substance, -: absence of the respective substance).

Differential gene expression induced by erlotinib

To further characterize the underlying molecular mechanisms of erlotinib-induced apoptosis and cell cycle arrest, the differential expression of genes related to cell cycle and apoptosis control was investigated using cDNA array technology. HepG2 cells were incubated for 48 h with 10 μmol/L erlotinib, as a significant arrest of both the cell cycle and apoptosis-induction had been observed under these conditions[12]. Erlotinib modulated the expression of 25 genes (Table 1). We found an overexpression of genes encoding apoptosis-related cysteine proteases (caspases) 3 and 7, both known to be important enzymes of the apoptotic process. Moreover, growth arrest and DNA-damage inducible (gadd-) genes encoding gadds 45 and 153 both associated with induction of apoptosis and growth arrest[15,16] as well as the insulin-like growth factor

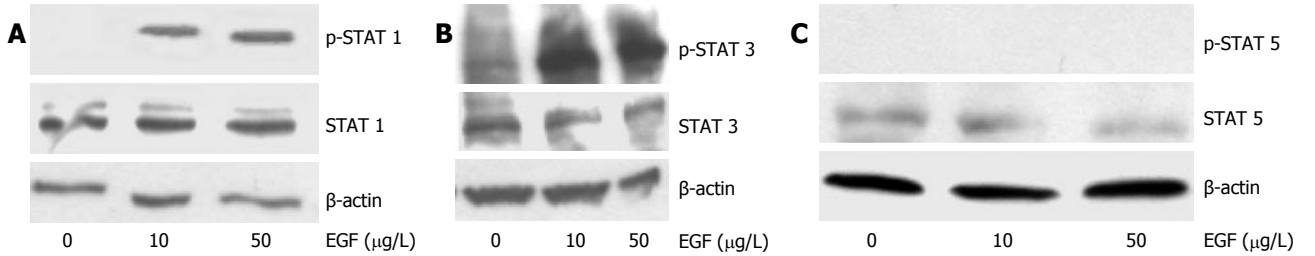

Figure 3 STAT-expression and EGF-induced activation in HCC cells. EGF induced STAT activation could be shown for STATs 1 and 3 (**A** and **B**) but not in the case of STAT5 (**C**).

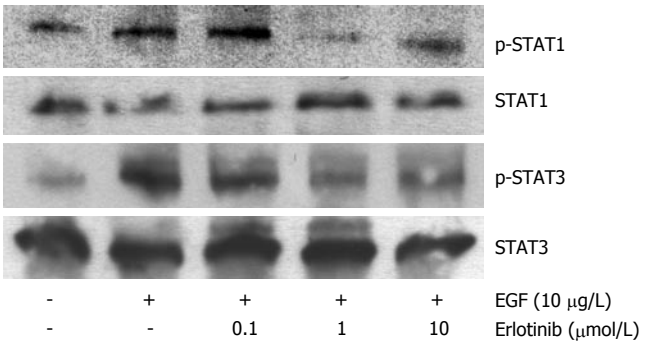

Figure 4 Erlotinib inhibited STAT-activation in HCC cells.

binding protein 6 (IGFBP-6) and cyclin B1, an important molecular regulator of the G_1/S and G_2/M cell cycle transitions[17] were markedly overexpressed. Genes encoding cell-cycle progression promoting proteins were found to be suppressed (particularly those proteins promoting the transition from the G_1 to the S-phase, e.g. CDK4 or cyclin A2). Additionally, a suppression of anti-apoptotic genes like Bcl-2 or the jun D proto-oncogene was detected as well as of the DNA-replication promoting proliferating cell nuclear antigen (PCNA).

Modulatory effect of erlotinib on expression of cell cycle regulators and antiapoptotic members of the Bcl-2 family

Changes in the expression of important cell cycle and apoptosis regulating proteins due to EGFR-inhibition were assessed by Western blotting. Treating HepG2 cells for up to 48 h with erlotinib (10 μmol/L) resulted in an increase of the cyclin-dependent kinase inhibitors (CDKIs) $p21^{Waf1/CIP1}$ and $p27^{Kip1}$. The expression of cyclin D1, a protein regulating the transition from the G_1 to the S-phase remained unchanged. Alterations in protein expression of the respective cell cycle regulators occurred within 24 h (Figure 5A). Additionally, a 48-h-treatment of HepG2 cells with 10 μmol/L erlotinib led to a significant suppression of the antiapoptotic Bcl-2 family members Bcl-X_L (Figure 5B) and Bcl-2 (Figure 5C).

Modulation of the MAP-kinase pathway and transactivation of EGFR induced by IGF-1

Comparable to the EGFR, the IGF-1-receptor is associated with carcinogenesis and tumor growth. Thus, we evaluated the mitogenic effects of IGF-1 on Huh-7 and HepG2 cells, both cell lines strongly expressing the IGF-1R[18]. Besides, we focused on a possible transactivation of the EGFR in response to IGF-1 treatment. Serum-starved HepG2 cells were treated for 15 min with IGF-1 (50-250 μg/L) followed by the determination of changes in EGFR- and ERK1/2-activation by Western blot. IGF-1 dose-dependently activated the mitogenic MAP-kinase pathway, moreover we could show that IGF-1 treatment in the absence of EGF or other growth factors resulted in an activation of the EGF-receptor, indicating a possible IGF-1R/EGFR cross talk in hepatocellular carcinoma cells (Figure 6).

Table 1 Transcripts differentially regulated in HepG2 cells in response to erlotinib

GenBankID	Gene name	Mean[1]	SD
M62402	Insulin-like growth factor binding protein 6	5.85	0.73
S66431	Retinoblastoma-binding protein 2	3.22	0.39
U13737	Caspase 3. apoptosis-related cysteine protease	3.18	0.52
Y09392	Tumor necrosis factor receptor superfamily. member 12 (translocating chain-association membrane protein)	3.04	0.61
M60974	Gadd45	2.37	0.34
S40706	Gadd153	2.31	0.09
M25753	Cyclin B1	2.21	0.47
U37448	Caspase 7. apoptosis-related cysteine protease	2.10	0.34
L16785	Non-metastatic cells 2. protein (NM23B)	2.03	0.22
U23765	BCL2-antagonist/killer 1	0.53	0.13
X51688	Cyclin A2	0.50	0.32
M34065	Cell division cycle 25C	0.49	0.06
L22005	Cell division cycle 34	0.48	0.17
X85134	Retinoblastoma-binding protein 5	0.38	0.12
L25676	Cyclin-dependent kinase 9 (CDC2-related kinase)	0.38	0.05
U66879	BCL2-antagonist of cell death	0.36	0.05
D25216	KIAA0014 gene product	0.35	0.14
X86779	Fas-activated serine/threonine kinase	0.30	0.06
L29220	CDC-like kinase 3	0.28	0.03
X74262	Retinoblastoma-binding protein 4	0.27	0.09
M14505	Cyclin-dependent kinase 4	0.25	0.07
AF010312	LPS-induced TNF-alpha factor	0.24	0.06
U25265	Mitogen-activated protein kinase 5	0.22	0.03
M15796	Proliferating cell nuclear antigen	0.17	0.03
X56681	Jun D proto-oncogene	0.13	0.06

[1] Arithmetic means of ratios (treated:untreated) from three separate array measurements.

Inhibitory action of erlotinib on mitogenic effects of EGF and/or IGF-1 and EGFR/IGF-1R receptor cross-talk

Erlotinib-pretreated (30 min, 10 μmol/L) serum-starved

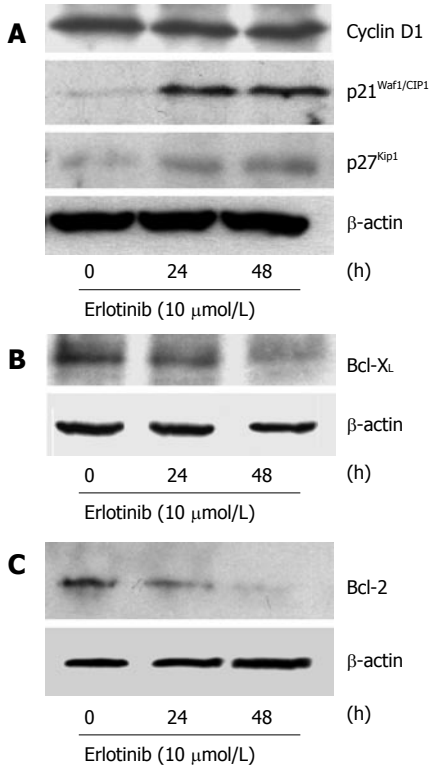

Figure 5 Erlotinib modulated the expression of cell cycle regulators (**A**) and antiapoptotic members of the Bcl-2 family (**B** and **C**).

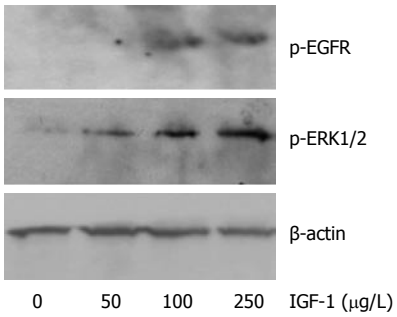

Figure 6 IGF-1-induced activation of ERK1/2 and transactivation of the EGFR.

HepG2 cells were incubated with EGF (10 μg/L) and/or IGF-1 (100 μg/L) for 15 min. Growth factor untreated cells served as control. Comparable to the experiments described above, EGF and/or IGF-1-treatment resulted in the activation of both EGFR and ERK1/2. Erlotinib pretreatment completely inhibited growth factor-induced EGFR activation and reduced p-ERK1/2 levels below control values. Thus, in addition to the blockade of EGF and IGF-1-induced mitogenic signaling, erlotinib potently suppressed IGF-1 induced IGF-1R/EGF-receptor transactivation (Figure 7). In order to exclude unspecific effects of erlotinib on the activation of the IGF-1R by its ligands, we additionally investigated the effects of the EGFR-blocker on the expression and activation of IGF-1R. HepG2 cells were cultured up to 48 h in medium containing 100 mL/L FCS and 10 μmol/L erlotinib. Western blot analysis showed neither changes in the expression of the β-chain of IGF-1R or the IGF-1R

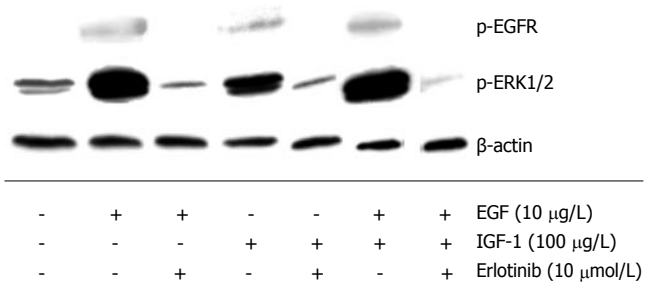

Figure 7 Erlotinib inhibited EGF and/or IGF-1-induced ERK1/2-activation and EGFR/IGF-1R cross talk.

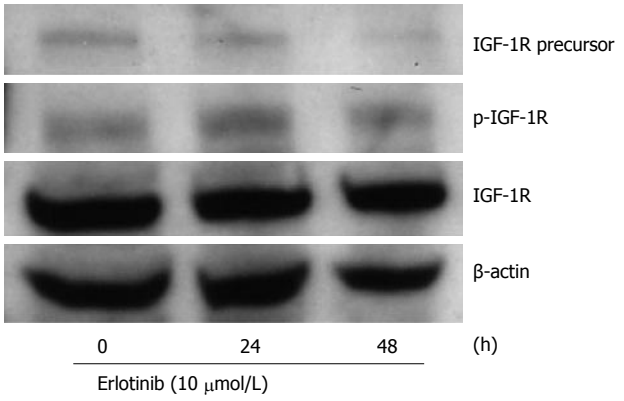

Figure 8 Erlotinib did not influence IGF-1R expression or activation.

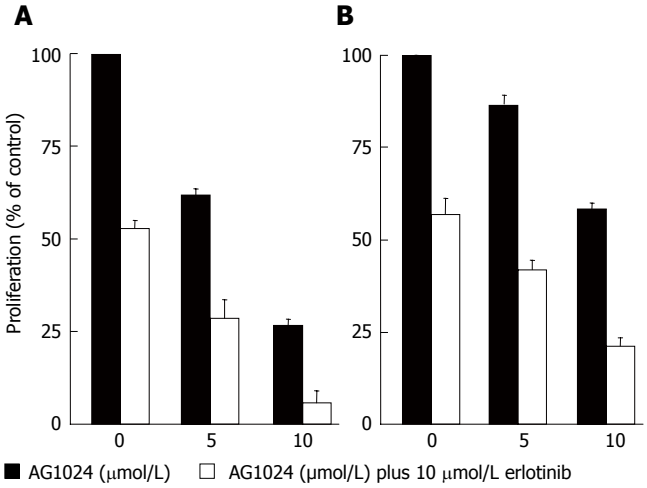

Figure 9 Antiproliferative effects of erlotinib plus AG1024. **A**: HepG2; **B**: Huh-7.

precursor nor in the phosphorylation of the receptor as compared to control (Figure 8).

Growth inhibitory effects of simultaneous blockade of EGFR and IGF-1R

On the basis of the results obtained in our investigations on EGFR/IGF-1R receptor signaling, we evaluated the growth inhibitory effects of a simultaneous blockade of the EGFR by erlotinib and the IGF-1R by AG1024. The tyrphostine AG1024 is a specific inhibitor of the IGF-1R-tyrosine kinase activity. Cells were treated with 5 or 10 μmol/L AG1024 and 10 μmol/L erlotinib for

72 h. Upon treatment with the IGF-1R-TKI alone both HepG2 (Figure 9A) and Huh-7 cells (Figure 9B) displayed a pronounced reduction of cell numbers (Figure 9, black bars). When combining AG1024 with erlotinib, synergistic antineoplastic effects were observed in both cell lines (Figure 9, hatched bars).

DISCUSSION

Treatment options of advanced hepatocellular cancer (HCC) are unsatisfactory, and the prognosis of patients suffering from advanced HCC is poor. New, effective and well-tolerated therapy strategies are urgently needed. The EGF/EGFR system is known to have strong stimulatory effects on the growth of hepatoma cells. Several studies have demonstrated EGFR expression to be a common feature of HCCs[4,5], underlining the role of the EGFR-TK as a rational target for future hepatocellular cancer treatment. We recently demonstrated that the EGFR-TK-inhibitor erlotinib potently inhibited the growth of EGFR-expressing human hepatocellular cancer cells by a time- and dose-dependent induction of apoptosis and an arrest at the G_1-to-S-transition of the cell cycle[12]. In accordance, preliminary results of a phase II trial of erlotinib in patients with HCC suggest a clinical benefit by erlotinib[19]. However, the underlying mechanisms and corresponding molecular events by which erlotinib mediates apoptosis and cell cycle arrest are not yet understood. In this study, we identified signaling molecules involved in erlotinib-mediated apoptosis and cell cycle modulations and showed the functional involvement of the identified pathways.

We demonstrate that EGFR is activated upon EGF binding in HCC cells with a subsequent activation of ERK1/2, a key protein of the mitogen-activated protein kinase (MAPK)-pathway and that erlotinib potently inhibits EGFR activation associated with an inhibition of the mitogenic downstream signaling. MAPKs are important regulators of apoptosis, proliferation and differentiation[20]. Once activated, ERK1/2 translocates to the nucleus where it acts as a regulator of gene expression of various proteins, e.g. activated ERK1/2 inhibits the expression of the cell cycle inhibitors $p21^{Waf1/CIP1}$ [21,22] and $p27^{Kip1}$ [23]. Using Western blotting we could demonstrate an increase of expression of both proteins in response to erlotinib treatment within 24 h. Moreover, ERK1/2-inhibition is known to up-regulate the expression of gadd45[24] which could be confirmed in this work by cDNA expression arrays. The expression of the gadd45 gene has been correlated with the presence of a strong growth arrest as it interacts with $p21^{Waf1/CIP1}$ to induce cell cycle arrest[25]. Overexpression of gadd genes causes growth inhibition and/or apoptosis, and combined overexpression of gadd genes leads to synergistic suppression of cell growth[26]. Furthermore, activated ERK1/2 has been reported to inhibit the apoptotic process by inhibiting caspase activation[27] and the expression of several antiapoptotic proteins[20]. In accordance, our previous investigations demonstrated the increase of caspase-3 activity due to EGFR-blockade[12,28]. In this work cDNA expression arrays revealed that gene expression of caspases 3 and 7 is up-regulated due to erlotinib treatment. In addition to the activation of the proapoptotic caspase network a decrease of gene and protein expression of antiapoptotic members of the Bcl-2 family as demonstrated by cDNA expression arrays and Western blotting may account for erlotinib's apoptosis inducing capabilities.

Comparable to the MAPK-pathway, Jak-STAT-signaling is involved in cell proliferation and cell cycle progression[29]. STATs are latent in the cytoplasm and become activated through tyrosine phosphorylation which typically occurs through JAKs or growth factor receptor-TKs. Phosphorylated STATs form homo- or hetero-dimers, enter the nucleus and function as transcription factors. Transcriptional changes concerning apoptosis and cell cycle related genes are similar to those described for ERK1/2[30]. In normal cells, ligand dependent activation of STATs is a transient process but in tumors the STAT proteins (in particular STATs 1, 3 and 5) are often constitutively activated[29]. STATs 3 and 5 are noted for the proliferative effects and inhibition of apoptosis whereas the role of STAT1 in oncogenesis and tumor progression is controversial[31]. Effects of EGFR-TK-inhibition on Jak-STAT-signaling has not been investigated so far. We demonstrated the expression of STATs 1, 3 and 5 in HepG2 cells and the activation of STATs 1 and 3 but not STAT5 due to EGF-stimulation. Erlotinib-treatment inhibited STAT-activation thus contributing to cell cycle arrest and apoptosis-induction.

To shed light on transcriptional changes in response to EGFR-TK-inhibition by erlotinib we performed cDNA expression arrays. As described above, erlotinib increased the expression of genes encoding proapoptotic factors like caspases and gadds whereas the expression of genes encoding antiapoptotic proteins like Bcl-2 or the jun D proto-oncogene was found to be decreased. At the same time we found a different expression of a variety of genes encoding cell cycle regulators: Cell-cycle promoters like CDK4 or cyclin A2 were suppressed, the important molecular regulator of the G_2/M cell cycle transition cyclin B1[17] was markedly overexpressed probably accounting for a partial G_2/M-block we observed in response to EGFR-blockade in previous investigations[12,28]. Interestingly, the insulin-like growth factor binding protein 6 (IGFBP-6) was found to be the gene with the strongest overexpression. IGFBPs are a family of six homologous proteins with high binding affinity for IGF-1 and IGF-2. In addition to functioning as simple carrier proteins, IGFBPs in serum regulate the endocrine actions of IGFs by changing the amount of IGF available to activate IGFRs, and locally produced IGFBPs act as autocrine/paracrine regulators of IGF action[32]. Furthermore, recent *in vitro* and *in vivo* findings show that IGFBPs may function independently of the IGFs as growth modulators[32]. IGFBP-6 differs from the other IGFBPs because it has a markedly higher affinity for IGF-2 than for IGF-1, whereas the other IGFBPs bind the two IGFs with similar affinities[33]. IGF-2 overexpression is described in several tumor-xenograft models and in human HCCs[34]. Additionally, a correlation of IGF-2 overexpression with HepG2 and Huh-7 cell growth has been shown[35] as well as a modulation of IGFBP-expression through the EGFR signaling pathway[36]. It may be speculated that reduced amounts of bioavailable IGF-2 as a result of EGFR-blockade-induced IGFBP-overexpression contribute to the growth inhibition of hepatocellular can-

cer cells by a further reduction of mitogenic stimuli. However, additional investigations on the complex network of IGF-2R, IGF-2 and IGFBPs have to be undertaken in order to explain our observations and their relevance with anti-EGFR based therapy strategies.

Some of the molecular targets investigated in this study may be used as surrogate biomarkers for anti-EGFR-based therapeutic strategies. The rational selection of cancer patients for EGFR inhibition therapies remains a major challenge because there is no clear correlation between EGFR overexpression and response to EGFR inhibitors[10]. Thus, the finding of new biomarkers is mandatory. Interestingly, using gadd153 induction as a predictor of clinical response has already been evaluated for paclitaxel treatment of cancer patients[37].

Like the EGFR, the insulin-like growth factor receptor 1 (IGF-1R) contributes to the growth, survival, adhesion and motility of cancer cells. The IGF-1R is a tetrameric tyrosine kinase receptor which can be activated by either IGF-1 or IGF-2. IGF-1R signaling is mediated through MAPK, phosphatidylinositol-3-kinase (PI3K) and stress-activated protein kinase (SAPK)[38]. In the present study we showed that the MAPK-pathway was activated by IGF-1 in HCC cells. Moreover, our results revealed an IGF-1R mediated transactivation of the EGFR in HCC cells. Several modes of indirect EGFR activation have been described so far[39]. As compelling evidence demonstrates the significance of EGFR signal transactivation in human disorders, the components of this signaling mechanism represent promising targets for therapeutic intervention. EGFR transactivation induced by activation of G-protein-coupled receptors, cytokine receptors and voltage-dependent Ca^{2+}-channels has been described. Though the exact mechanisms of the receptor-receptor cross talk are not known yet, RTKs are supposed to be important mediators of the transactivation process[40]. In accordance, our results exhibited erlotinib's potency to inhibit IGF-1 induced transactivation without affecting the activation of the IGF-1R by its ligands. This finding may explain results of previous *in-vitro* studies showing greater antineoplastic activity for EGFR-TK-inhibition in HepG2 and Huh-7 cells[12,18] than for inhibition of endogenous ligand binding by cetuximab[28].

In addition to the induction of EGFR-transactivation, IGF-1R is known to be involved in resistance towards anti-EGFR-based therapeutic approaches. This arises from the fact that alternative signaling pathways of the IGF-1R can compensate for a blocked primary EGFR pathway. As IGF-1R is strongly expressed in HCC cells[34], co-targeting of IGF-1R and EGFR in HCC cells may be a way to avoid or overcome resistance towards EGFR blockade. Combining erlotinib with the IGF-1R-TKI AG1024 resulted in synergistic effects in HepG2 and Huh-7 cells. These results suggest that combination regimens targeting both EGFR and other growth factor receptors such as IGF-1R may simultaneously yield greater anticancer activity than approaches that address only a single receptor and should be investigated more extensively in future studies.

In summary, our data suggest that EGFR-TK-blockade by erlotinib leads to an inhibition of the mitogenic MAP-kinase pathway as well as to an interruption of STAT-mediated signaling, resulting in a different expression of apoptosis and cell cycle regulating genes in HCC cells. Overexpression of proapoptotic factors like caspases and gadds associated with a down-regulation of antiapoptotic factors like Bcl-2, Bcl-X_L or jun D may account for erlotinib's action to induce apoptosis. Downregulation of cell cycle regulators promoting the G1-to-S-transition and overexpression of cyclin-dependent kinase inhibitors and gadds contribute to the induction of a G_1/G_0-arrest in response to EGFR-TK-inhibition by erlotinib. Moreover, our results point at the transactivation of EGFR-mediated signaling by the IGF-1R and show erlotinib's inhibitory effects on the receptor-receptor cross talk mechanisms. Finally we demonstrate that synchronous targeting of EGFR and IGF-1R yields greater antineoplastic effects than approaches that address only a single receptor. To conclude, our study sheds light on the understanding of the mechanisms of action of EGFR-TK-inhibition in HCC-cells and thus might facilitate the finding of combination therapies that act additively or synergistically. Moreover, our data on the pathways activated by erlotinib could be helpful in predicting the responsiveness of tumors to EGFR-TKIs in the future.

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BASIC RESEARCH

5-Fluorouracil-related enhancement of adenoviral infection is Coxsackievirus-adenovirus receptor independent and associated with morphological changes in lipid membranes

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Abstract

AIM: To evaluate the mechanism underlying the effects of 5-Fluorouracil (5-FU) on adenoviral infection.

METHODS: Low and high Coxsackievirus-Adenovirus Receptor (CAR) expressing human colon carcinoma cell lines were treated with 5-FU and two E1-deleted adenoviral constructs, one transferring GFP (Ad/CMV-GFP) the other bax (Ad/CEA-bax). The number of infected cells were monitored by GFP expression. To evaluate the effects of 5-FU in a receptor free system, Ad/GFP were encapsulated in liposomes and treated with 5-FU. Ad/GFP release was estimated with PCR and infection of 293 cells with the supernatant. Electron microscopy of the Ad5-GFP-liposome complex was made to investigate morphological changes of the liposomes after 5-FU.

RESULTS: Infection rates of all cell lines increased from 50% to 98% with emerging 5-FU concentrations. The enhanced viral uptake was independent of the CAR expression. Additionally, 5-FU treated liposomes released 2-2.5 times more adenoviruses. Furthermore, 5-FU-treated liposomes appeared irregular and porous-like.

CONCLUSION: adenoviral uptake is enhanced in the presence of 5-FU irrespective of CAR and is associated with morphological changes in membranes making the combination of both a promising option in gene therapy.

Key words: 5-Fluorouracil; Coxsackievirus-adenovirus receptor; Adenoviral infection; Gene therapy

INTRODUCTION

It is widely accepted that cancer is the endpoint of an accumulation of genetic mutations that result in a cellular phenotype characterized by uncontrolled growth and reduced apoptosis. Consequently, therapeutic strategies, which address the genetic lesions and thus kill cancer cells, are reasonable. This concept has made virus-mediated gene therapy an ideal candidate for therapeutic approaches either alternative or complementary to chemotherapy or radiotherapy. Human adenoviruses are widely used as delivery systems but adenovirus (Ad)[3] uptake is dependent on expression of Coxsackievirus-adenovirus receptor (CAR). Unfortunately, tumor cells are usually characterized by a reduced expression of CAR that binds the fiber knob domain of the Ad serotypes 2 and 5[1]. Cells lacking this receptor are more resistant to adenoviral infection and, consequently, they are poor targets for Ad-associated tumor therapies[2-5]. Further, it has been shown that the treatment of colorectal cancer with the replication-selective Ad *dl*1520 in combination with 5-Fluorouracil (5-FU) was more efficient in inducing apoptosis than the administration of the two agents separately[6-9]. Therefore we evaluated the mechanism underlying the effects of 5-FU in the context of adenoviral infection in this study. To better understand the role of 5-FU in adenoviral infection of tumor cells, we used two E1- and replication-deficient adenoviral mutants expressing GFP (Ad-GFP) to infect colorectal cancer cell lines that show different CAR expression. A significantly higher number of GFP-expressing cells were observed after treatment with 5-FU and Ad-GFP compared to Ad-GFP alone. The effect of 5-FU was even more striking in a cell line with low CAR expression (SW480) indicating that a CAR-independent mechanism may be responsible for the transport of Ad through the cell membrane. This enhancement

of infection was dose-dependent and maximal with simultaneous application of 5-FU and Ad-GFP. To assess this effect in a CAR-independent pathway, Ad-GFP was encapsulated in liposomes, which were treated with 5-FU. Supernatants of these liposomes contained 2.4 times more Ad-GFP after 5-FU treatment compared to controls. In addition, morphological changes in the lipid membranes were seen by electron microscopy. In conclusion, we could demonstrate that simultaneous treatment with 5-FU enhances adenoviral uptake into tumor cells. More importantly, this effect could be observed irrespective of CAR expression and 5-FU favors the crossing of the viral protein capsid through the lipid membranes. Regardless of the underlying mechanism, the combination of adenoviral and 5-FU treatment might be of significant importance in the context of gene transfer.

MATERIALS AND METHODS

Cell lines and culture conditions

All cell lines used in this study were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and were grown in the appropriate media containing 100 mL/L FCS, 10 g/L Pen/Strep (PS) and 10 g/L glutamine. The human embryonic kidney 293 cells were grown in DMEM, the human colon adenocarcinoma cells DLD-1, LOVO, SW480 and SW620 in RPMI 1640, and the RKO cells in McCoy.

Recombinant virus construction and purification

The adenoviral vectors for gene expression were constructed with the AdEasy system[10]. Briefly, Ad-CEAbax was constructed with the pAdTrack vector containing the CEA promoter in front of the bax gene and the gene for GFP under the control of the CMV promoter, whereas Ad-GFP was constructed with the pAdTrack vector containing GFP under the control of the CMV promoter. The resulting plasmids were then transformed into *Escherichia coli* cells with pAdEasy-1. The recombinant adenoviruses were generated in the 293 cells (E1-transformed) and purified by CsCl gradient ultracentrifugation[11]. The titer was determined by counting the green cells after 48 h.

Cell culture and infection

For Ad infection of SW480 the cells were seeded onto 96-well plates at 1500 cells/well and grown for 2 d. For the 5-FU pretreatment, the cells were first incubated at 37℃ in RPMI/FCS/PS containing 5-FU at concentrations of 2, 10, 30 and 50 μmol/L. After 2 h the medium was removed, the cells were washed and infected with Ad-GFP at 300 pfu/well in 200 μL medium without the drug. For the co-treatment, the cells were infected with Ad-GFP diluted in 200 μL medium containing 5-FU at the concentrations reported above. Controls were incubated with medium without 5-FU. The number of green cells was counted after 24, 48 and 72 h with a fluorescence microscope for GFP expression. For the infection of the Lovo and SW480 cells with Ad-CEAbax, the cells were plated onto 60 mm dishes at a density of 2×10^5 cells/dish. After 1 d, the cells were infected with the virus (1 MOI) in 1 mL medium without FCS and PS. After 30 min incubation at 37℃, the cells were treated with the medium containing FCS, PS and 5-FU at the final concentrations of 2 μmol/L for Lovo and 20 μmol/L for SW480. For the treatment of the colon cancer cells with Ad-GFP and 5-FU, 5-BrU, 5-FC, DOC, or taxol, the cells were seeded onto 6-well plates at 2×10^5 cells/well. After 1 d, Ad-GFP was added at 3×10^4 pfu/well in 1 mL medium without FCS and PS. After 30 min incubation at 37℃, 2 mL growth medium were added containing FCS, PS and the drug at the following final concentration: 4 μmol/L for 5-FU, 5-BrU, 5-FC and DOC, and 5 μg/L for taxol. The number of green cells was counted 48 h after infection.

Liposome preparation

To assess the effects of 5-FU of lipid membranes and the ability of Ad to penetrate the membranes, we encapsulated Ad in liposomes with and without 5-FU. The release of Ad was measured by PCR for E4orf6. In addition, the number of infective particles was evaluated by infection of 293 cells with the supernatant. The liposomal formulation of Ad was prepared as follows: Ad-GFP (3×10^4 pfu) was mixed with lipofectamine (Invitrogen) that was brought to a final concentration of 0.4 g/L with PBS. After 2 h incubation at room temperature, 5-FU was added to the final concentration of 20 μmol/L. The mixture was incubated overnight at room temperature and then centrifuged at 2060 g for 50 min at 20℃. The supernatant (100 μL/well) was diluted to 1 mL with DMEM without FCS and PS and added to the cells that were then incubated for 30 min at 37℃. Afterwards, 2.5 mL DMEM containing FCS and PS were added and the cells were further incubated. For the experiment with the liposome-encapsulated Ad-GFP, the 293 cells were plated into 6-well plates at 2×10^5 cells/well and were grown overnight.

PCR analysis

The following adenoviral DNA samples (each 2 μg) were prepared for PCR: DNA alone, DNA in the presence of 9.6 μmol/L 5-FU, DNA/lipofectamine (0.2 g/L), DNA/lipofectamine (0.2 g/L) in the presence of 9.6 μmol/L 5-FU. The samples were incubated overnight at room temperature, then centrifuged at 2060 g for 35 min at 18℃. For control, an additional sample of DNA/lipofectamine (0.2 g/L) was prepared and treated with a lysis buffer for 1 h at 4℃ prior to centrifugation. The DNA was precipitated from the supernatants upon addition of NaOAc and isopropanol, isolated by centrifugation, washed with 750 mL/L ethanol and dried. The PCR was performed by using the Taq PCR Master Mix (Qiagen). The resultant PCR products were then resolved on a 1.5% agarose gel containing 0.25 mg/L of ethidium bromide.

Flow cytometric analysis

Single cell suspensions were fixed in 700 mL/L ethanol and incubated with 50 g/L PI and 20 g/L RNAse for 15 min. at 37℃. Flow analysis was done at 488 nm excitation and > 525 nm Em range collected for GFP fluorescence. Elite Software 4.0 (Coulter Corp, Miami, FL) and Multi

Cycle DNA Analysis program software (Phoenix Flow Systems, San Diego, CA).

Western blot analyses

Protein concentration was determined by using the BC assay (Interchim, Montluçon, France). The protein samples [(40 μg for the CAR detection, 60 μg for the bax detection and 50 μg for the fiber detection (samples were not cooked)] were separated on SDS-polyacrylamide gels (100 g/L for the CAR detection, 150 g/L for the bax detection and 75 g/L for the fiber detection), and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The blots were blocked with 50 mL/L nonfat dry milk in 1 g/L Tween 20 PBS (TPBS-MLK) for 1 h at room temperature, then incubated overnight at 4℃ with the appropriated antibodies: CAR (goat polyclonal antibody, Santa Cruz) at the dilution of 1:285, bax (polyclonal rabbit anti-human, PharMingen) at the dilution of 1:1000, fiber (polyclonal mouse, NeoMarkers, Fremont, CA) at the dilution of 1:1000 and actin (goat polyclonal antibody, Santa Cruz) at the dilution of 1:1000. After washing with TPBS, the following secondary antibodies were added at the dilution of 1:500 and incubated for 1 h at room temperature: HRP-conjugated IgG from donkey anti-goat for CAR and actin detection, and from goat anti-rabbit for bax detection. The proteins were finally visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

CAR blockade

SW480 cells were plated into 6 well plates at 2×10^5 cells/well and incubated with CAR antibody (goat polyclonal antibody, Santa Cruz) at the dilution of 1:1000, 1:750, 1:500, 1:250, 1:100 and 1:50 for 24 h. The cells were incubated with Ad-GFP and the number of green cells was counted 48 h after infection.

Transmission electron microscopy

The following solutions were analyzed by electron microscopy (EM): (A) lipofectamine at 0.5 g/L, (B) lipofectamine at 0.5 g/L treated overnight with 20 μmol/L 5-FU, (C) Ad-GFP (4×10^8 pfu/L) complexed with lipofectamine at 0.5 g/L, (D) Ad-GFP (4×10^5 pfu/mL) complexed with lipofectamine at 0.5 g/L and incubated for 1 h at room temperature prior to treatment overnight with 20 μmol/L 5-FU. The EM samples were prepared by using the negative stain procedure. Briefly, a drop of each sample was deposited on a copper grid and coated by a formvar/carbon film. The film was then stained with 20 g/L tungsten phosphoric acid and dried on air. The EM images were recorded on a Zeiss instrument operating at 80 kV.

RESULTS

5-FU augments the viral infection rate of both high and low CAR-expressing cell lines

Several cell lines (DLD-1, Lovo, SW480 and SW620) were treated with Ad-GFP or Ad-CEAbax at a MOI that infected 50% of the cells. The infection of all cell lines was surprisingly efficient (up to 98%) when the adenoviral constructs were used in combination with 5-FU, as indicated by the high population of green cells in Lovo and SW480 cell examples (Figure 1A and 2A). In accordance with GFP expression, an increased expression of bax was detected (Figure 1B) after Ad-CEAbax infection and 5-FU. The RKO cells died after simultaneous treatment and thus the number of green cells decreased after 24 h (Figure 4). As control 293 cells were transfected with the CMV Promoter/GFP DNA and treated with 10 μmol/L 5-FU. No difference in the amount of green cells was seen after 5-FU indicating that 5-FU does not interfere with the transcription of the reporter gene (data not shown). In addition, SW480 cell lysates of 5-FU treated (10 μmol/L) and control cells were blotted for adenoviral fiber protein after treatment with two different adenoviral MOI (200 and 50). In both cases more fiber protein could be detected in 5-FU treated cells (Figure 2B) indicating a higher amount of intracellular adenovirus. 5-FU enhanced the uptake of Ad not only in cell systems expressing CAR at high levels, such as the Lovo cells, but even more in cells with a low-level CAR expression (Figure 1C), indicating that the effect might not be dependent on CAR expression. To block the function of CAR SW480 cells were incubated with

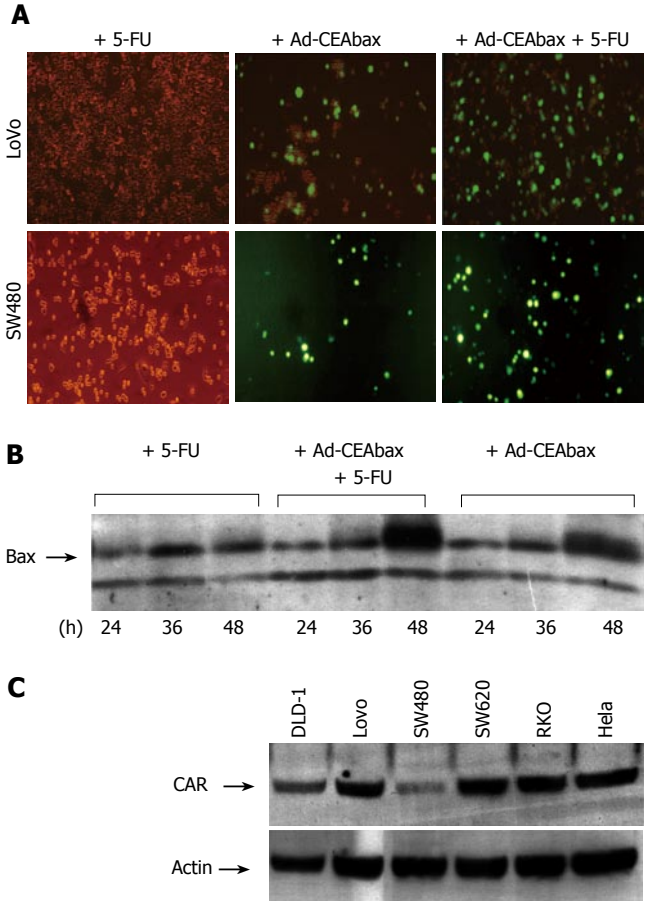

Figure 1 Effect of 5-FU on the adenoviral infection of colon carcinoma cell lines differing in CAR expression. The drug was used at the dose of 2 μmol/L for the Lovo cells and of 20 μmol/L for the SW480 cells. The virus (Ad-CEAbax) was used at 1 MOI. The cells were treated with 5-FU alone, or infected with the virus in the absence and in the presence of 5-FU (**A**). The expression of bax with 5-FU and/or the virus was controlled by western blot after 24 h, 36 h and 48 h incubation (**B**). The expression level of CAR on the colon carcinoma cells line was confirmed by western blot analysis (the expression of actin is reported as a control for loading) (**C**).

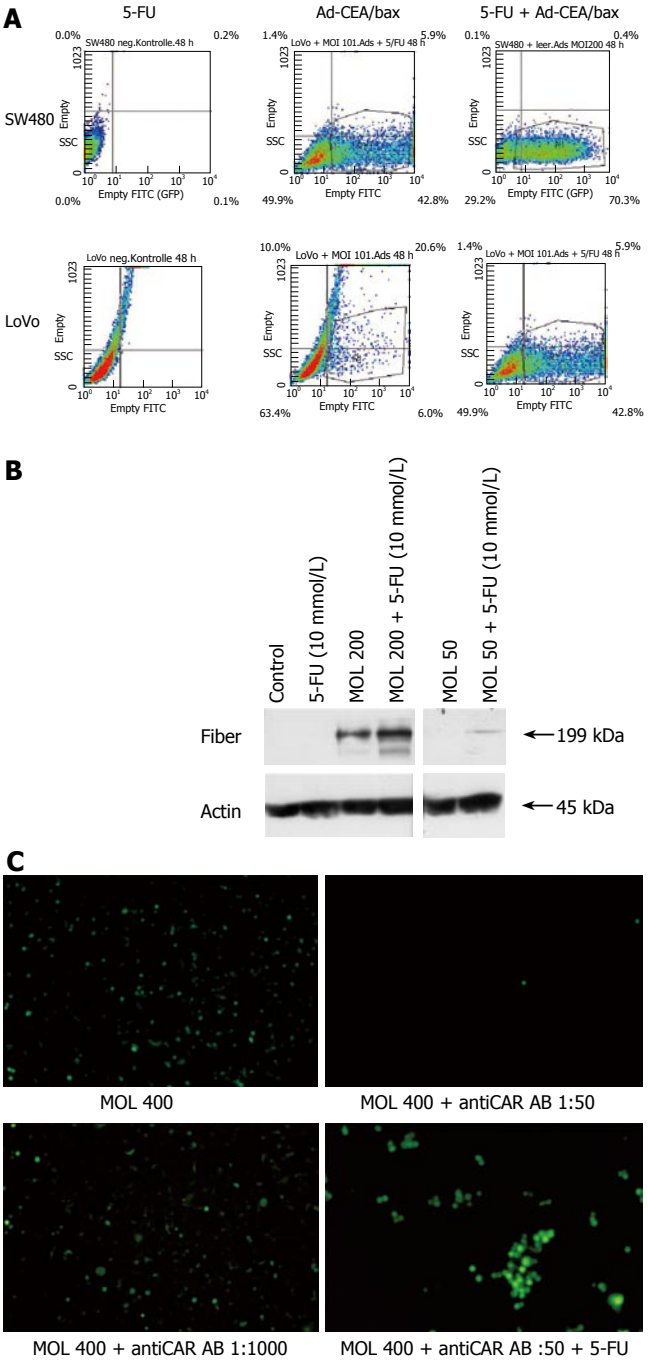

Figure 2 The number of GFP expression cells was measured by flow cytometry in SW480 and Lovo cells (**A**). Treatment with 5-FU resulted in increased number of GFP expression cells and increased intensity of GFP expression in both cell lines. Intracellular adenoviral fiber protein was assessed by western blot analysis of SW480 cell lysates (**B**) after Ad-GFP treatment. In control cells and 5-FU treated cells no fiber protein could be detected. Low amounts of fiber protein could be seen in Ad-GFP treated cells dependent on Ad-GFP concentrations (MOI of 200 and 50). Additional 5-FU treatment increased fiber protein in whole cell lysates indicating enhanced adenoviral uptake. Function of CAR was blocked by anti CAR antibody at a concentration of 1:500 but 5-FU enhanced the number of GFP expressing cells even at anti CAR antibody concentrations of 1:50 (**C**).

antibody against CAR (Santa Crux, N-17) in increasing concentrations from 1:1000 to 1:50 for 24 h and incubated with Ad (MOI 400). Viral uptake was blocked at higher concentrations than 1:500 but treatment with 5-FU still enhanced viral uptake (Figure 2C) accounting for a CAR independent mechanism of 5-FU.

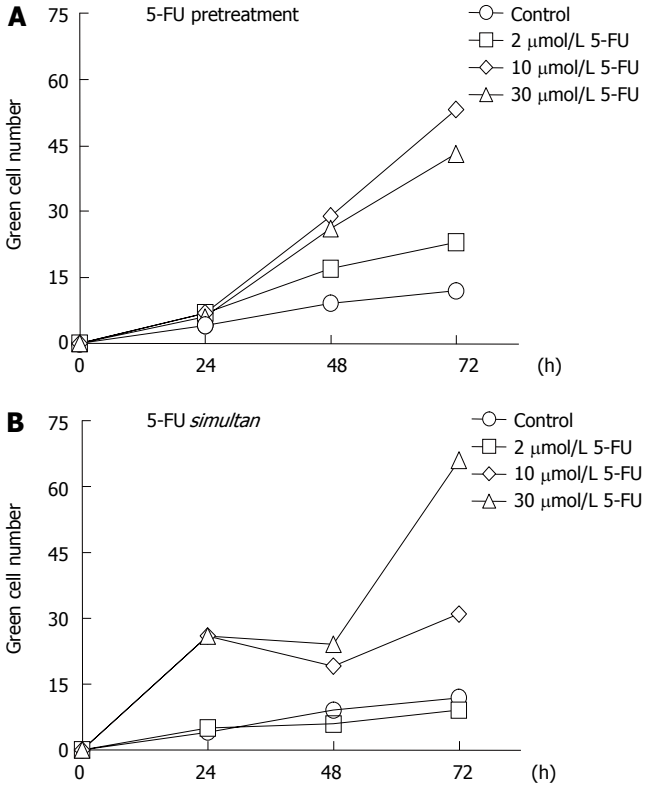

Figure 3 Dependence of adenoviral efficacy on the sequencing of 5-FU and virus administration to the SW480 cells. Two h postincubation with the drug at the indicated concentrations, the drug was removed and the cells were infected with the adenoviral construct Ad-GFP (**A**). Alternatively, the cells were infected with the virus in combination with 5-FU (**B**). The number of green cells was counted after 24, 48 and 72 h infection. The reported data points are the average of three experiments and the standard deviation values were in the range of 10%-15% (the error bars were not reported for clarity).

Efficacy of 5-FU on Ad uptake is dependent on drug concentration

The GFP expression in 5-FU-pretreated SW480 cells was significantly higher than in untreated cells, and the number of cells producing the fluorescent protein increased with increasing concentrations of the anticancer agent up to 10 μmol/L. Indeed, after 48 and 72 h the rate of Ad infection in cell pretreated with 10 μmol/L 5-FU was more than twice the infection rate obtained with 2 μmol/L 5-FU (Figure 3A). At the higher concentrations of 30 μmol/L, however, the drug was shown to be moderately less effective, a phenomenon that could be due to an inhibitory effect of 5-FU on the viral DNA replication (data not shown). All experiments were done in triplicate and the standard deviation was less then 15%. A superior efficacy of 5-FU was observed by its simultaneous application with Ad, which led to an improvement not only in the yield but also in the rate of the infection, especially when the drug was used at 30 μmol/L. Under these conditions the number of green cells counted after 24 h was three times higher than in the case of the drug pretreatment and there was an increment of more than 50% in the density of green cells after 72 h (Figure 3B).

The increase in cellular Ad uptake is a specific response to 5-FU

The positive effect of 5-FU on Ad infection could be

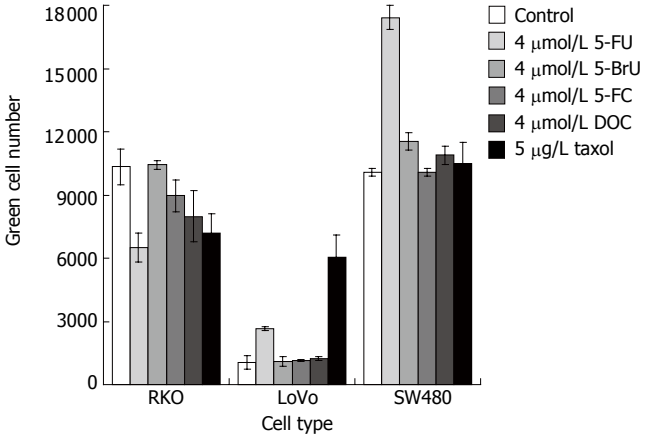

Figure 4 Other pyrimidine-based drugs do not show the positive effect of 5-FU on adenoviral infection. The colon carcinoma cell lines were treated concurrently with the virus Ad-GFP and 5-FU, 5-BrU, 5-FC, DOC or taxol, each at the indicated concentration. The number of the infected green cells was counted after 40 h infection. The reported data are the average of three experiments and the error bars indicate the standard deviation values.

related to its function as a chemotherapeutic agent or to its structural and chemical characteristics. To assess which structural and chemical features of 5-FU might play a role in the entry of Ad into the cells, the two compounds 5-BrU and 5-FC, which are pyrimidine derivatives as 5-FU, were used in combination with Ad. In 5-BrU the halogen is bigger and less electronegative than in 5-FU. In 5-FC an amino group is present at position 4 instead of a carbonyl group as in 5-FU, thus conferring a higher hydrophilicity to the molecule. In both cases, the level of the infection did not change with respect to Ad alone, indicating that the ability of 5-FU to favor Ad infection is specific and not common to other molecules structurally-related to 5-FU. Additionally to other chemotherapy drugs, such as taxol[12], the cell membrane-destabilizing bile salt sodium deoxycholate[13] was used in combination with Ad. In the case of the DLD-1 and SW480 cells only 5-FU positively influenced the cellular entry of Ad, whereas taxol did not show any effect. In contrast, the infection of the Lovo cells was improved by both anticancer drugs, with taxol being twice more effective than 5-FU (Figure 4). The effect of these drugs was only moderate in the RKO cells, which do express high levels of CAR.

5-FU increases the release of Ad from liposomal formulations

Based on the observation that 5-FU positively affected the Ad uptake independently of CAR, we postulated a 5-FU-mediated transfer of Ad through lipid membranes. To investigate whether 5-FU exerts any effect on ordered lipid structures, a liposome mixture of DOSPA/DOPE at a 3:1 ratio was used to encapsulate the Ad, and the resulting complex was then treated with 5-FU overnight. After centrifugation, the number of infective particles in the supernatant was tested on 293 cells, which provide a system for the replication of E1B deleted Ad mutants. As shown in Figure 5A, the green cell number obtained from the infection with the supernatant of the 5-FU-treated Ad-liposome preparation was at least 2.4 times higher than that obtained from the infection with the supernatant of the same preparation but without 5-FU. This is indicative of an augmented viral concentration in the supernatant as the result of the incubation of the liposome-entrapped Ad with 5-FU. In order to control whether the treatment with 5-FU could induce the release of adenoviral DNA from the liposomes, samples of DNA-liposome solutions with and without 5-FU were subjected to PCR and then analyzed by agarose gel electrophoresis. No adenoviral DNA was visualized by ethidium bromide staining, indicating that there was no release of the DNA component from the liposome complex in the presence of 5-FU. As a control, the DNA-liposome complex was subjected to liposome disruption prior to PCR and the resultant PCR product was visualized on the agarose gel, as it was expected (Figure 5B).

5-FU induces a morphological change of the lipid layers

The EM images of the liposomal formulations upon 5-FU treatment showed a multilayer motif that was characterized by an irregular thickness of the liposome as a result of the disappearance of an ordered layer structure in some regions (Figure 5C). Similarly, in the case of the Ad-liposome complex treated with 5-FU, the lipid surface was not uniform, but showed some bright spots that are probably indicative of perturbations in the packing and ordering of the multilayers.

DISCUSSION

In this study we could demonstrate that 5-FU increases the effectiveness of adenoviral uptake into colorectal cancer cells, thus overcoming the resistance of several colorectal cancer cells to adenoviral treatment. This effect is independent of CAR expression on the cell surface and could be confirmed in a receptor free system. In addition this effect is associated with changes in lipid membranes. Thus, the combination of the anti-tumor drug 5-FU with adenovirus enhances gene transfer capabilities of the virus in colon cancer cells. The synergistic effect of oncolytic Ad (*dl*1520) and chemotherapeutic agents, such as 5-FU and cisplatin[6-9], is already known, but the reason for such behavior is not completely elucidated. One proposed explanation is the enhancement of cell chemosensitivity induced by viral replication, probably through the expression of the E1A gene that occurs after Ad infection and increases tumor cell killing[14]. Nevertheless, this mechanism does not apply to all cell systems, because it can occur only in those cells that are not resistant to the Ad entry and, more strikingly, our data demonstrate that even E1-deleted constructs, which are replication-deficient, are more effective in the context of 5-FU treatment.

As stated before, a replication defect of human Ad was used to transfer CMV promoter/GFP DNA but 5-FU could probably increase transcription of the CMV promoter similar to FDXR induction by p53 in cells treated with 5-FU[15]. Therefore we transfected cells with CMV promoter/GFP plasmid and could not see any enhancement of GFP expression with of 5-FU. In addition, higher amounts of adenoviral fiber protein were detected in cell lysates after Ad and 5-FU treatment indicating more Ad particles within the cells

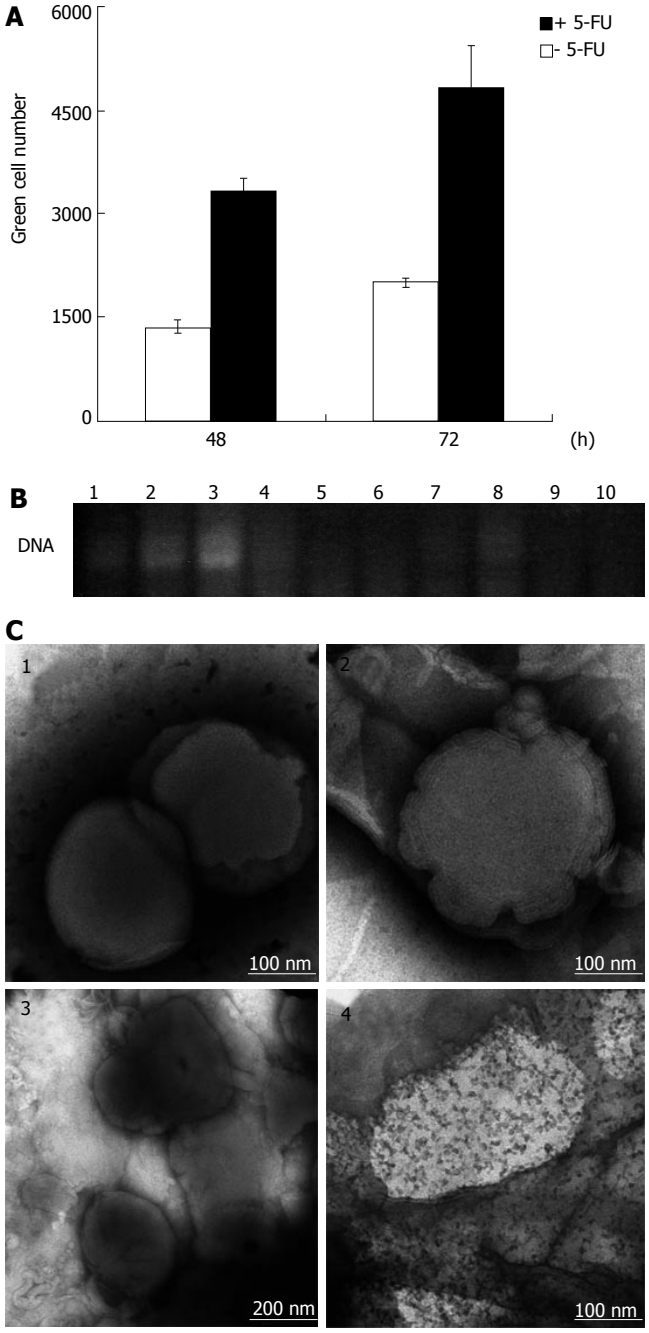

Figure 5 Increased release of viral particles from liposome-encapsulated adenoviral formulations. 293 cells were infected with the supernatant from centrifuged liposome-entrapped Ad-GFP solutions with and without 5-FU incubation. The number of the infected green cells was counted after 48 h and 72 h infection. The reported data are the average of three experiments and the error bars indicate the standard deviation values (**A**). On the other hand, the 5-FU treatment of liposome-encapsulated viral DNA did not lead to any DNA release, as confirmed by PCR analysis. Following samples (each loaded in duplicate) were separated by agarose gel electrophoresis: DNA alone (lanes 1 and 2), DNA and 9.6 μmol/L 5-FU (lanes 3 and 4), DNA and lipofectamine (lanes 5 and 6), DNA with lipofectamine and lysis buffer (lanes 7 and 8), DNA with lipofectamine and 5-FU 9.6 μmol/L (lanes 9 and 10) (**B**). EM images of liposomal preparations. (1) lipofectamine, (2) lipofectamine after treatment overnight with 5-FU, (3) Ad-GFP complexed with lipofectamine, (4) Ad-GFP complexed with lipofectamine after treatment overnight with 5-FU (**C**).

and suggesting that the higher amount of green cells is a result of higher Ad uptake into cells. Furthermore, it was proposed that the loss of sensitivity of cancer cells to Ad treatment is generally a consequence of loss of CAR representing a severe limitation of the application of gene therapeutic strategies with Ad as a gene delivery system[11]. Interestingly, although the binding of Ad to its receptor is suggested to be the first step for infection, our results indicated that the presence of CAR does not provide a guarantee for efficient viral infection. Indeed, not only cells expressing low levels of CAR, such as the SW480 cells, but also cells expressing normal levels of CAR, such as the DLD-1, Lovo and SW620 cells, were found to be Ad-resistant. However, simultaneous treatment with 5-FU and Ad could enhance the sensitivity to adenoviral infection in all tested cell systems but the effects of 5-FU treatment was more impressing in cell lines which are difficult to infect with Ad. To assess the effect of 5-FU we blocked CAR and could still see enhanced viral uptake. Interestingly, the positive effect of 5-FU was superior when both Ad and 5-FU were added simultaneously in comparison to the pre-incubation of the cells with the drug before the addition of Ad. This suggests that the entry of Ad into cells may occur independently of the production or degradation of effectors caused by 5-FU treatment. Therefore we assessed the capability of Ad to pass through liposomal membranes in the presence of 5-FU and, in accordance with this hypothesis, we observed an increased adenoviral release from liposomes treated with 5-FU.

In light of our experiments on cells differing in CAR expression, we postulate that Ad uptake could be based on a mechanism alternative to that requiring the binding to the Ad receptor. In the presence of CAR, Ad is delivered into the cell via an internalization process involving receptor-mediated endocytosis[16]. Alternatively, in the absence of CAR, the adenoviral protein capsid is likely to interact directly with the phospholipid layers of the cell membrane; however, such interaction results in an effective intracellular transfer only in the presence of 5-FU. The intra- and extra-cellular drug diffusion across the membrane could be coupled to temporary changes in the packing and ordering of the lipid bilayers building the cell membrane, which, in turn, could become more accessible to external agents. Nevertheless, the potential effects of 5-FU on the membrane must be different from those of other amphiphylic molecules that are known to exert a lytic action on membranes, such as DOC[17], as suggested by the observation that this bile salt did not increase the Ad uptake. Previous studies have reported on morphological changes of cells after treatment with adenoviruses[18] or with detergents[17]. Moreover, the interactions between biological or model membranes and hydrophobic drugs, such as 1, 4-dihydropyridines[19] and benzocaine[20], have been investigated in detail, but the exact mechanism of 5-FU with cell membranes remains elusive. The human colon cancer cells tested in this work generally became bigger and adopted a spindle shape upon incubation with 5-FU. This supports our hypothesis that, beside its well-known anti-cancer action, 5-FU may exert a potential disturbing effect at the cell membrane. Interestingly, 5-FU changes intestinal absorption in rat of dextran[21]. However, the exact mechanism of adenoviral passage through lipid membrane in the presence of 5-FU remains unclear.

In conclusion, we suggest that 5-FU might play a role

in increasing the sensitivity of cells for environmental influences by changes in the phospholipid bilayers of cell membranes in addition to its chemotherapeutic property. This would be especially useful for the transport of therapeutic compounds independently of the presence or absence of specific cell surface receptors, as in the case of the transfer of Ad into CAR-negative cells. On the other hand, side effects of adenoviral infections during high-dose chemotherapy might not only be based on a suppressed immune system, but, if 5-FU enhances viral uptake, flue-like side effects of the 5-FU chemotherapy might be caused by a higher adenoviral uptake into cells.

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BASIC RESEARCH

Involvement of P53 and Bax/Bad triggering apoptosis in thioacetamide-induced hepatic epithelial cells

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Abstract

AIM: Thioacetamide (TAA) has been used in studying liver fibrosis and cirrhosis, however, the mechanisms of TAA-induced apoptosis in liver are still unclear. The hepatic epithelial cell line clone 9 was cultured and treated with TAA to investigate the causes of cell death.

METHODS: The cell viability of TAA-induced clone 9 cells was determined using MTT assay. Total cellular GSH in TAA-induced clone 9 cells was measured using a slight modification of the Tietze assay. The activity of caspase 3 in TAA-induced clone 9 cells was monitored by the cleavage of DEVD-p-nitroanaline. TUNEL assay and flow cytometry were applied for the determination of DNA fragmentation and the proportion of apoptosis in TAA-induced clone 9 cells, respectively. The alterations of caspase 3, Bad, Bax and Phospho-P53 contents in TAA-induced clone 9 cells were measured by Western blot.

RESULTS: The experimental data indicated that TAA caused rat hepatic epithelial cell line clone 9 cell death in a dose-and time-dependent manner; 60% of the cells died (MTT assay) within 24 h after 100 mg/L TAA was applied. Apoptotic cell percentage (TUNEL assay) and caspase 3 activities were highest after 100 mg/L TAA was added for 8 h. The release of GSH and the elevation in caspase content after TAA treatment resulted in clone 9 cell apoptosis *via* oxidative stress and a caspase-dependent mechanism. The phospho-p53, Bax and Bad protein expressions in clone 9 cells were increased after TAA treatment.

CONCLUSION: These results reveal that TAA activates p53, increases caspase 3, Bax and Bad protein contents, perhaps causing the release of cytochrome c from mitochondria and the disintegration of membranes, leading to apoptosis of cells.

INTRODUCTION

Thioacetamide (TAA) is employed as a curing ingredient, a chemical reagent, a raw medicine, a pesticide, a textile dye and a finishing auxiliary. TAA[1-4] is a typical hepatotoxin that causes centrilobular necrosis similar to carbon tetrachloride[5-8] and D-galactosamine[9]. TAA-induced hepatotoxicity *via* its S-oxide metabolite (thioacetamide-S-dioxide), that interferes with the motion of RNA from the nucleus to the cytoplasm, resulting in structural and functional cellular deformation and leading to membrane injury. TAA induces hepatocyte damage following its metabolism to thioacetamide sulphene and sulphone, *via* a critical pathway that involves CYP4502E1-mediated biotransformation[10,11]. TAA is a well-known hepatotoxicant whose administration to rodents *in vivo* causes cell death *via* both apoptosis and necrosis[2,12,13]. The observed liver enzyme activities of aspartate and alanine aminotransferases (AST, ALT), glutamate dehydrogenase and threonine deaminase were low in the TAA-treated group. The declines were significant for both transaminases and threonine deaminase[14]. TAA (200 μg/mL)-incubated hepatocyte cells exhibited a 40%-62% reduction in the marker enzymes (AST, ALT, and alkaline phosphatase (AP)) and a 50%-61% reduction in both viability and O_2 uptake[15]. It may reduce the amount of antioxidant reagents, including vitamin C, vitamin E and glutathione contents[16].

Elevated levels of reactive oxygen species (ROS) are believed to mediate damage by their interaction with proteins, nucleic acids, carbohydrates and lipids[17-19], changing the enzymatic function and forming oxidation products. ROS is maintained at physiologically optimal levels under normal conditions by antioxidant defense systems that contain nonenzymatic antioxidants-glutathione and enzymes such as superoxide dismutases

(SOD), catalase and glutathione peroxidase (GPX)[20]. The reduction in the antioxidant defenses and the reduced scavenging capacity reported herein may contribute to oxidative stress in rats with hemorrhagic shock and can be explained by various factors. The observations of apoptosis in liver are based on histochemical changes[2], the participation of ROS[21] and lipid peroxidation[22-24], and cysteine-aspartate proteases3 (caspase 3) activation[16,25] following the administration of TAA. However, the mechanisms of TAA-induced liver injury are not yet completely understood. The rat liver cell line, clone 9, is an epithelial cell line isolated from a young male rat in 1968 and has primarily been used for studies of *in vitro* carcinogenesis and toxicology. This study was to investigate whether TAA-induced oxidative stresses caused cell death and to seek the pathway that is involved in TAA-induced apoptosis using the rat hepatic epithelial cell line clone 9 as a model and to understand the regulatory mechanisms of TAA-induced liver injury.

MATERIALS AND METHODS

The rat hepatic epithelial cell line clone 9 (CRL-1439, ATCC, USA) was cultured with DMEM/F-12 medium to which 10% fetal bovine serum was added in 50 ml/L CO_2 at 37℃.

Experimental treatments

In Expt1, 5×10^5 clone 9 cells were cultured for 12-16 h in 6 wells and the medium was changed. Afterward, 25, 100 or 200 mg/L TAA was added to the wells and incubated for various times to determine the cell viability (MTT assay). In order to get a large enough cell pellet for chemical assay, the density of cultured cells was changed to 2×10^6 and cells were cultured in 25 cm^2 flasks. In Expt2, 2×10^6 clone 9 cells were cultured for 12-16 h in 25 cm^2 flask and the medium was changed. Then, 100 mg/L TAA was added to the flask and incubated for various periods to measure caspase 3, Bad, Bax and Phospho-P53 contents (Western blot), as well as total GSH and caspase 3 activity. In Expt3, 2×10^6 clone 9 cells were cultured for 12-16 h in a 25 cm^2 flask and the medium was changed. Thereafter, 100 mg/L TAA was added to the flask and incubated for various times to examine DNA fragmentation (TUNEL assay) and measure the proportion of apoptosis (flow cytometry).

MTT assay

750 μL of MTT (5 mg/mL, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) solution was added to each well and incubated for 4 h. 1 mL of a DMSO working solution (180 mL DMSO with 20 mL 1 N HCl) was added to each well, and the OD of the yellow reaction product was evaluated in an ELISA reader at a wavelength of 570 nm with a reference wavelength of 630 nm after 15 min.

TUNEL assay

DNA strand breaks were identified using the Boehringer TUNEL assay (Boehringer Mannheim, Marburg, Germany). Briefly, cells in each experiment were washed with phosphate-buffered saline (PBS). The cells were fixed and permeabilized using the Starfiqs IQP-200 reagents (Immuno Quality Products, Groningen, The Netherlands) according to the manufacturer's instructions. Subsequently, the TUNEL reaction mixture (60 min, 37℃) was added and the samples were washed and analyzed using FACSort flow cytometry (Becton Dickinson, New Jersey, USA). The cells that expressed FITC fluorescence were considered to be TUNEL positive. This experiment was performed separately: the observed numbers of apoptotic cells could not be compared directly with those counted in the other experiments. Additionally, this experiment was conducted after a culturing period of 48 h.

Caspase 3 activity assay

The caspase 3 assay was performed according to the method of Hampton *et al*, 2002[26]. Briefly, cells were treated with lysis buffer (100 mmol/L Hepes, 10% sucrose, 5 mmol/L dithiothreitol and 0.1% Chaps at pH 7.25). The extracted lysates were incubated with DEVD-p-nitroanaline for 3 h at 37℃. Caspase 3-dependent cleavage of p-nitroanaline (pNA) was monitored at 405 nm by spectrophotometry. Caspase 3 activity is expressed in pmol/min per μg protein.

Glutathione assay

Total cellular GSH was measured using a slight modification[27,28] of the Tietze assay [5]. The Tietze assay is a sensitive and specific method for determining the amounts of both reduced (GSH) and oxidized (GSSG) forms of glutathione in unknown samples[28]. A stock buffer solution of 0.1 mmol/L sodium phosphate, pH 7.5, with 1 mmol/L EDTA was prepared in distilled water and utilized to make separate solutions of 12 mmol/L NADPH, 0.1 mmol/L DTNB and 50 U/mL GSH reductase. The assay was carried out directly in 96-well plates. 100 μL DTNB, 20 μL GSH reductase and 20 μL NADPH were added to each well. Absorbance at 405 nm with a reference wavelength of 595 nm was measured for 30 min at room temperature, using an automatic multiwell microplate spectrophotometer (Beckman, Opsys, MR, USA), and final values were calculated as a mean of three readings. The automatic mix function on the microplate reader was utilized to shake the plate before each reading. Standards of known GSSH content were prepared by serial dilution in 1x MES buffer and used to construct a standard curve. The rate of increase in absorbance at each concentration of GSSH standard was linear throughout the 1.5 min assay. The total protein concentration of each sample was determined using a Bio-Rad kit[29] with bovine serum albumin as standard, such that GSH levels could be expressed as μmol per μg protein. All determinations were carried out in multiples of 12 wells.

Western blotting

After treatment, the cells were washed in D-PBS and lysed with protein lysis buffer (137 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$ and 0.1 mmol/L sodium orthovanadate, containing 1% Nonidet P-40 and 1 mmol/L phenylmercuric sulfonyl chloride). The lysate was centrifuged at 12000 r/min for 10 min at 4℃ and the supernatant was collected. The protein

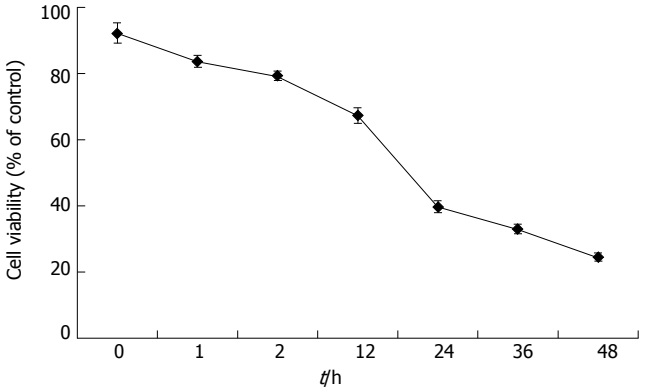

Figure 1 Cell viability of clone 9 after treatment with 100 mg/L TAA for various periods. The results were obtained using MTT assay. Data are presented as mean ± SE. Individual experiment was repeated three times and each time point of treatment was triplicate.

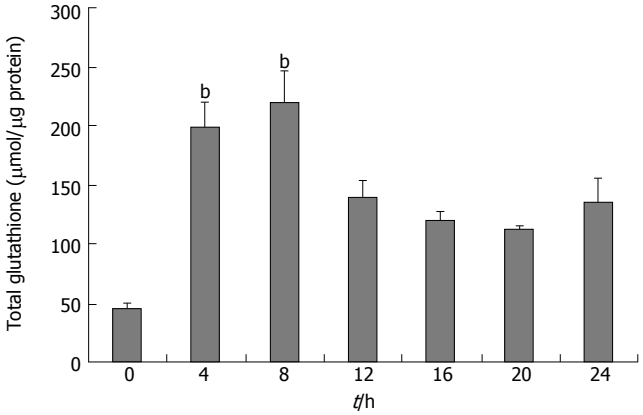

Figure 2 Total glutathione (GSH and GSSG) of clone 9 after treatment with 100 mg/L TAA for various times. Data are presented as mean ± SE. $^{b}P < 0.001$ *vs* the control (0 h). Individual experiments were repeated three times and each time point of treatment was triplicated.

content was determined using Bradford assay (BioRad) with BSA as a standard. The supernatant (total protein 20 μg) was mixed with 6x electrophoresis sample buffer that contained 1, 4-dithiothreithol (DTT). The proteins were separated by electrophoresis on a 10% gradient polyacrylamide slab gel and were electrophoretically transferred to a PVDF membrane (Amersham Life Science, Buckinghamshire, UK). The blots were incubated overnight in 3% BSA /TBS-T buffer (50 mmol/L Tris-HCl, 2.7 mmol/L KCl, 0.01 mol/L phosphate, 0.09% NaCl, pH 7.5 and 0.05% Tween-20) for 2 h. Membranes were incubated for 12-16 h with rabbit anti-caspase 3 polyclonal antibody (in a 1:1000 dilution, MWt 35 kDa, Cat. #9665, Cell Signaling, MA, USA), rabbit anti-Bad polyclonal antibody (in a 1:2000 dilution, MWt 21.5 kDa, Cat. #9292, Cell Signaling), rabbit anti-Bax polyclonal antibody (in a 1:1000 dilution, MWt 20.5 kDa, Cat. #2772, Cell Signaling) or mouse anti-Phospho-P53 monoclonal antibody (in a 1:2000 dilution, MWt 53 kDa, Cat. #9286S, Cell Signaling). After three washes with PBST, the blot was incubated with AP-conjugated secondary antibody for at least 2 h and immunoreactive proteins were visualized using BCIP/NBT (ZYMED, 00-2209). The band intensities of the control and the TAA treatment were determined using PHORETIX (Feng Jin Biomedical & Instruments Co Ltd, 61397/28052 Memo-HASP1) and the TAA treatment was compared with the control following-actin normalization.

Statistical analysis

All data are presented as mean ± SE. For each measurement, data obtained at different times were compared statistically ($P < 0.05$) by one-way analysis of variance (ANOVA) with a Duncan multiple range test from SAS/STAT for multiple comparisons. A P value of less than 0.05 was considered to indicate a significant difference.

RESULTS

Cell viability

To determine the effective concentration of TAA, various concentrations of TAA were added to the clone 9 cell line, respectively. The cell survival rate fell to 67% and 40% at 12 and 24 h after 100 mg/L TAA-treated clone 9 was applied, respectively (Figure 1). Treatment with a low concentration (25 mg/L) of TAA did not cause significant cell death at 48 h (data not shown). Nevertheless, 200 mg/L TAA was too toxic for clone 9 cells and most cell death occurred within 2 h of treatment. Thus, 100 mg/L TAA was chosen for the subsequent experiments.

Total GSH content

The clone 9 cells were treated with 100 mg/L TAA for various times (4, 8, 12, 16, 20, and 24 h). The total glutathione (GSH plus GSSG) of cell homogenate was measured. The total GSH content of the 100 mg/L TAA-treated clone 9 cells was elevated at 4 h and reached a peak level at 8 h ($P < 0.05$). Afterward total GSH content of the 100 mg/L TAA-treated clone 9 cells declined from 12 h to 24 h compared to that of the TAA-treated 4 h (Figure 2).

Caspase 3

The clone 9 cells were treated with 100 mg/L TAA for various times (4, 8, 12, 16, 20, and 24 h). The caspase 3 activity and protein content (Western blot) of cell homogenate were determined. The caspase 3 activity of the 100 mg/L TAA-treated clone 9 cells reached a peak between 8 and 12 h (Figure 3A). The caspase 3 protein content of the 10 mmol/L TAA-treated clone 9 cells was gradually elevated from 4 h and reached a peak between 8 and 16 h (Figure 3B).

DNA fragmentation

The cell morphology was changed and irregularly shaped DNA was distributed close to the interior of the cell membrane, appearing like an apoptotic body (Figure 4A). Hoechst 33342 and TUNEL assay were applied to elucidate the type of cell death after TAA-treated clone 9 cells. The occurrence of apoptosis increased by 23% at 8 h after 100 mg/L TAA-treated clone 9 cells (Figure 4B).

Phospho-P53 expression

The clone 9 cells were treated with 100 mg/L TAA for various times (4, 8, 12, 16, 20, and 24 h). The phospho-P53

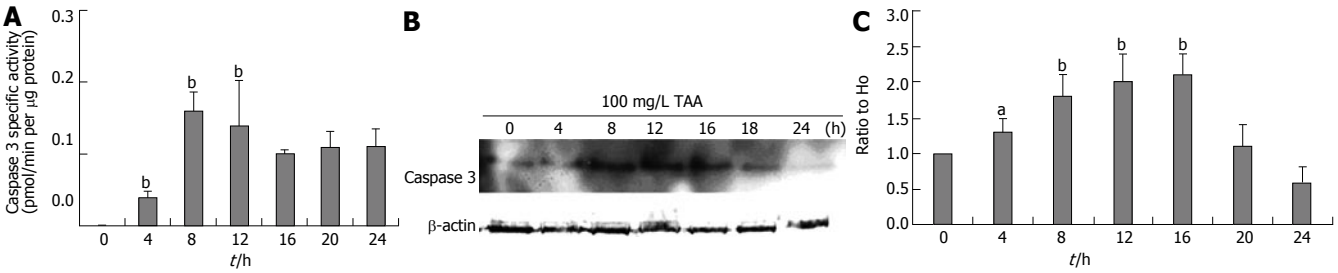

Figure 3 **A**: Caspase 3 activity; **B**: Caspase protein level (Western blot); **C**: Alterations of caspase protein compared to H0 (zero hour) after β-actin normalization (Western blot) in clone 9 after treatment with 100 mg/L TAA for various times. Data are presented as mean ± SE. $^{a}P < 0.05$ *vs* the control (0 h); $^{b}P < 0.01$ *vs* the control (0 h). Individual experiments were repeated three times and each time point of treatment was triplicate.

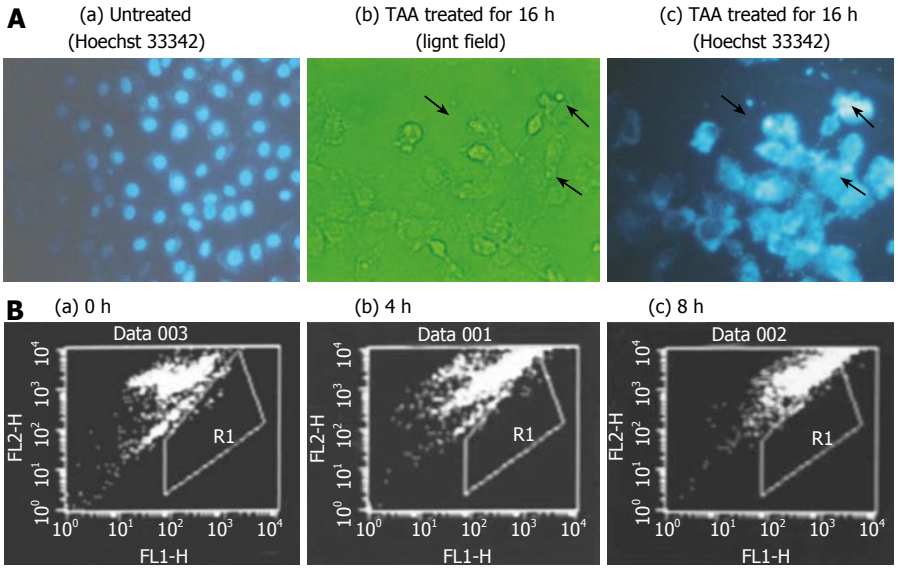

Figure 4 **A**: Morphology of clone 9 cell; (a) untreated (Hoechst 33342 staining); (b) after treatment with 100 mg/L TAA treatment for 16 h (light field); (c) after treatment with 100 mg/L mmol/L TAA for 16 h (Hoechst 33342, 5 mg/mL in PBS). The arrow indicates the apoptotic cells; **B**: Flow cytometry of apoptosis in clone 9 cells (TUNEL assay) after treatment with 100 mg/L TAA for various times (a) 0 (b) 4 and (c) 8 h. R1 presents the area of cell apoptosis. Individual experiments were repeated three times and each time point of treatment was triplicate.

protein (Western blot) of cell homogenate was determined. The phospho-P53 expression of 100 mg/L TAA-treated clone 9 cells was increased at 4 h and maintained to 8 and 12 h ($P < 0.05$) (Figure 5).

Bax and Bad expressions

The clone 9 cells were treated with 100 mg/L TAA for various times (4, 8, 12, 16, 20, and 24 h). The Bax and Bad protein (Western blot) of the cell homogenate were determined. The Bax and Bad expressions of 100 mg/L TAA-treated clone 9 cells were elevated at 4 h ($P < 0.05$) and maintained to 8 and 12 h ($P < 0.05$) (Figures 6 and 7). These results were consistent with the phospho-P53 expression.

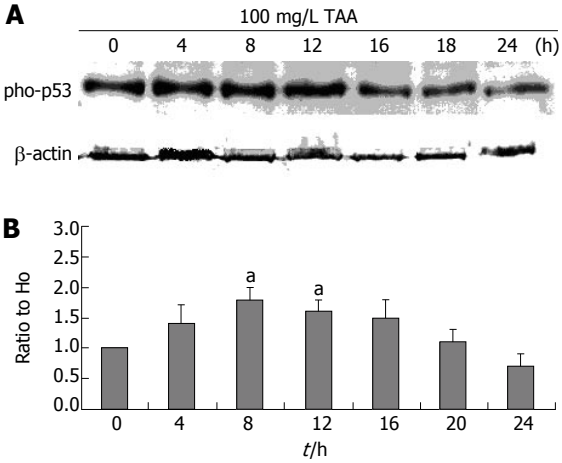

Figure 5 **A**: Phospho-p53 protein level (typical data, Western blot); **B**: Alterations of phospho-p53 protein as compared to H0 following β-actin normalization of clone 9 after treatment with 100 mg/L TAA for various times. Data are presented as mean ± SE. $^{a}P < 0.05$ *vs* the control (0 h). Individual experiments were repeated three times and each time point of treatment was triplicate.

DISCUSSION

In the present study, rat hepatic epithelial cell line clone 9 was found apoptotic following TAA-induction.

Total GSH

The binding of the reactive compound thioacetamide-S dioxide to tissue macromolecules may be responsible for hepatic necrosis, apoptosis[16], perturbations of mitochondrial activity[30,31] and the elevation of serum cytokine levels. Several studies of rats and cultured cells have shown the involvement of oxidative stress in the etiology of TAA-induced liver damage. In these works, TAA caused lipid peroxidation[29,32-35]; increased the susceptibility of hepatocytes to *in vitro* lipid peroxidation[23]; reduced the GSH/GSSG ratio[23,34,36,37], increased GSH

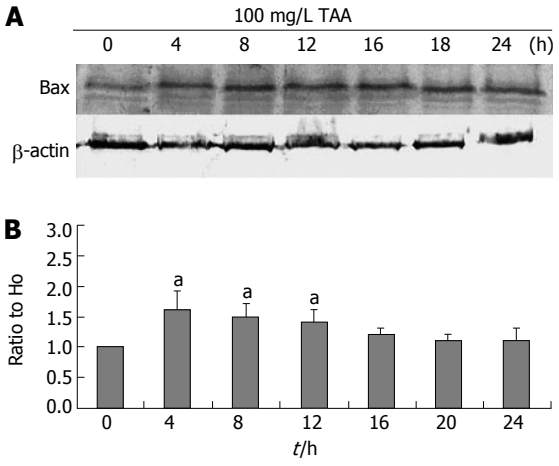

Figure 6 **A**: Bax protein level (typical data, Western blot); **B**: Alterations of Bax protein as compared to H0 following β-actin normalization of clone 9 after treatment for various times with 100 mg/L TAA. Data are presented as mean ± SE. $^aP < 0.05$ *vs* the control (0 h). Individual experiment was repeated three times and each time point of treatment was triplicate.

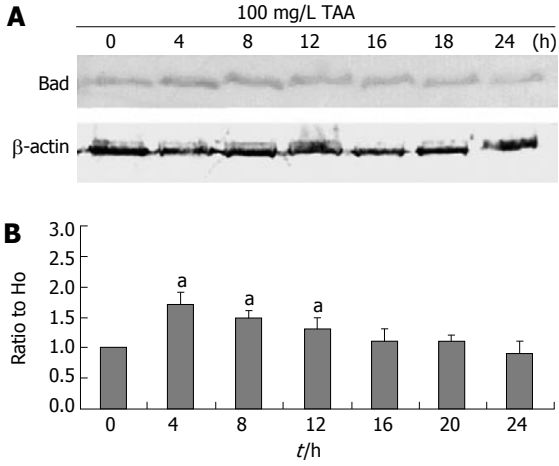

Figure 7 **A**: Bad protein level (typical data, Western blot); **B**: Alterations of Bad protein as compared to H0 following β-actin normalization of clone 9 after treatment for various times with 100 mg/L TAA. Data are presented as mean ± SE. $^aP < 0.05$ *vs* the control (0 h). Individual experiments were repeated three times and each time point of treatment was triplicate.

synthesis[33], alterations in low molecular weight[16] and the formation of enzymatic antioxidants[36-38]. The data herein revealed that the total GSH content of 100 mg/L TAA-treated clone 9 cells was elevated at 4 h, reached the highest level at 8 h and decreased from 12 to 24 h (Figure 2). The method in this work detects the total level of GSH. It addresses not only the origin of intracellular GSH and GSSG but also that of the new generation of GSH. Accordingly, total GSH represents the level of cell to anti-oxidative stress. TAA induces oxidative stress, causing the hepatocytes to have a high total GSH content.

Dependence of apoptosis on caspase 3

The discovery of a family of cysteine-aspartate proteases (caspases) and their participation in signaling and apoptosis indicates the critical importance of these enzymes in this form of cell death[39]. When cells are caused to undergo apoptosis, caspases-in particular caspase 3-cleave ICAD to dissociate the CAD: ICAD complex, allowing CAD to cleave chromosomal DNA[40]. This study tested caspase 3 protein expressions by western blotting and detected caspase 3 enzyme activity to determine the time point of hepatic apoptosis with TAA treatment. The data herein demonstrate that enzyme activity and western blotting both indicated that caspase 3 activity and the protein of caspase 3 in the 100 mg/L TAA-treated clone 9 cells peaked between 8 and 12 h. The apoptosis caused by TAA involved the activation of caspase 3.

Phospo-p53 and Bax/Bad

In the signaling process associated with apoptosis, the membrane integrity of mitochondria plays an important role and is regulated by the Bcl-2 protein. On the basis of structural and functional attributes, Bcl-2 proteins can be divided into three subgroups; (1) anti-apoptotic channel-forming Bcl-2 proteins with four BH domains (BH1 to -4) and a transmembrane anchor sequence, (2) proapoptotic channel-forming proteins with all but the BH4 domain (Bax, Bak and Bok) and (3) proapoptotic ligands (BAD, BOD/Bim and BID) that contain only the BH3 domain[41-43]. The first two subgroups of proteins are believed to be anchored on the mitochondrial membrane, while the third subgroup of proteins act as ligands that dimerize with the membrane-anchored, channel-forming Bcl-2 "receptors"[44,45]. The BH3 domains in the third subgroup are critical for the binding activity of these ligands. Proapoptotic BOK and BAX with a channel-forming domain regulate apoptosis by releasing APAF-1 because of suppression by anti-apoptotic proteins and by promoting the release of cytochrome c[46]. Most studies that compare Bax and cytochrome c proteins in cytosolic and mitochondria demonstrate the results of Bax translocation[47]. In this work, after TAA treatment, the total protein levels of Bax and Bad are elevated perhaps because the regulation of Bax genes increases the amount of Bax protein and the translocation into mitochondria, disintegrating the membrane and causing cell death. The increment of cytosolic Bad protein may be caused by phospho-Bad dephosphorylation; the dephosphorylated Bad and Bcl-XL then generates a dimer that influences the release of cytochrome c and, consequently, apoptosis[48]. DNA damage activates latent tetramers of p53 to bind in a loosely defined DNA recognition sequence within target gene regulatory elements; p53 then typically activates gene transcription[49,50]. The activation of DNA binding and subsequent transactivation activities of p53 occur *via* a phosphorylation-acetylation cascade[51]. The carboxyl-terminus of p53 is phosphorylated in response to DNA damage, as is the amino-terminus; the effects of the amino- and carboxyl-terminal modifications on p53 determine its downstream specificity. Overall, the combination of different phosphorylation and acetylation events that affect p53 tetramers probably determine binding preferences of the downstream target gene-response elements, causing DNA damage-induced signaling *via* p53 phosphorylation. In this work, the expression levels of phospho-p53 and Bax are increased 4 h after treatment with TAA. The apoptotic rate reached 23% at 8 h after treatment with

TAA. This difference may be caused by the activation by ROS of p53. The formation of free radicals and the increase in ROS by H_2O_2 constitute the mechanism of apoptosis[52-57]. The H_2O_2-induced apoptosis penetrates the cell membrane[58] and alters Ox-red and free OH-damaged DNA[59].

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CLINICAL RESEARCH

Wireless capsule endoscopy in the investigation of patients with chronic renal failure and obscure gastrointestinal bleeding (preliminary data)

Stephanos Karagiannis, Spyros Goulas, Georgios Kosmadakis, Petros Galanis, Dimitrios Arvanitis, John Boletis, Evangelos Georgiou, Christos Mavrogiannis

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Abstract

AIM: To investigate the role of wireless capsule endoscopy (WCE) in detection of small bowel (SB) pathology in patients with chronic renal failure (CRF) and obscure bleeding.

METHODS: Consecutive CRF patients with obscure bleeding were prospectively studied. Patients with normal renal function and obscure bleeding, investigated during the same period with WCE, were used for the interpretation of results.

RESULTS: Seventeen CRF patients (11 overt, 6 occult bleeding) and 51 patients (33 overt, 18 occult bleeding) with normal renal function were enrolled in this study. Positive SB findings were detected in 70.6% of CRF patients and in 41.2% of non-CRF patients ($P < 0.05$). SB angiodysplasia was identified in 47% of CRF patients and in 17.6% of non-CRF patients. Univariate logistic regression revealed CRF as a significant predictive factor for angiodysplasia ($P < 0.05$). Therapeutic measures were undertaken in 66% of the patients with the positive findings.

CONCLUSION: According to our preliminary results, SB angiodysplasia was found in an increased prevalence among CRF patients with obscure bleeding. WCE is useful in diagnosis of gastrointestinal pathologies and in planning appropriate therapeutic intervention and, therefore, should be included in the work-up of this group of patients.

Key words: Chronic renal failure; Obscure gastrointestinal bleeding; Wireless capsule endoscopy; Angiodysplasia

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INTRODUCTION

Unexplained gastrointestinal bleeding (overt or occult) and anemia are common complications of advanced chronic renal failure (CRF)[1,2]. Furthermore, 19% of patients with advanced CRF prior to dialysis and 6% of those on maintenance hemodialysis have hemoccult positive stool[3]. In some cases, upper and lower gastrointestinal tract endoscopic and imaging studies cannot explain these symptoms and findings and bleeding remains a real diagnostic and therapeutic challenge.

Until recently, small bowel (SB) investigation with the available endoscopic and imaging studies was incomplete. Consequently, there are no studies giving information on the pathology of the entire SB in CRF patients, except for two autopsy studies[4,5]. In view of the scarcity of information, we conducted this study and used wireless capsule endoscopy (WCE) to evaluate SB pathology in patients with advanced CRF and obscure bleeding. This novel method is well tolerated and allows complete visual investigation of the SB[6,7]. Moreover, the diagnostic yield of WCE, especially in obscure gastrointestinal bleeding, is significantly higher to that of any other methods, including push enteroscopy, small bowel radiography, computed tomography, magnetic resonance imaging and angiography[8]. To the best of our knowledge, this is the first endoscopic study to explore the entire SB in CRF patients with obscure gastrointestinal bleeding.

MATERIALS AND METHODS

This prospective ongoing study included consecutive patients referred to our institution with advanced CRF needing SB investigation for obscure bleeding. Advanced CRF was defined as creatinine clearance of less than 30 mL/min. Renal transplant recipients with stable renal function and serum creatinine concentration $\geqslant$ 2 mg/dL for at least a 6-mo period were eligible to enter the study as well.

Age- and sex-matched patients with normal renal function and obscure gastrointestinal bleeding, investigated with WCE during the same period, were used for the interpretation of CRF patients' findings.

Obscure gastrointestinal bleeding was defined according to the American Gastroenterological Association position statement[9]. Patients with obscure bleeding had been initially investigated with esophagogastroduodenosc opy and colonoscopy in other institutions and, if negative, were subsequently referred for WCE.

Generally accepted contraindications for the WCE procedure are described elsewhere[10]. Written informed consent was obtained from all patients. Patients' clinical characteristics, including sex, age, total duration of CRF (pre-treatment plus post treatment period), NSAID or aspirin use, comorbid diseases (practically any serious diseases, e.g. cardiovascular, collagen diseases, endocrinopathies, portal hypertension), need for previous transfusions as well as the etiology of renal failure were recorded. Biochemical tests of renal function (serum creatinine and urea) and hemoglobin level were obtained as well.

WCE procedure

M2A capsule (Given Imaging, Yoqneam, Israel) was used in this study. Patients' preparation and WCE procedure were carried out following the generally recommended guidelines[11]. Hemodialysis patients were not given the two-liter solution of polyethylene glycol as preparation, due to restriction of fluid intake; instead, they were advised to abstain from solid food, on the day before the procedure.

Interpretation of WCE results

A single gastroenterologist (experienced in gastrointestinal endoscopy and WCE) initially screened all videos and selected images of potential abnormalities. Then, two gastroenterologists (also experienced in interpreting WCE findings) independently reviewed the selected images to confirm the accuracy of diagnosis. All videos were extensively discussed and findings identified by both reviewers were considered as definitive and included in the report. The procedure was defined as incomplete if the capsule failed to pass into the cecum during an eight and a half-hour period of the examination. The diagnostic yield of WCE was calculated for both CRF and non-CRF patients. Only lesions with a high potential for bleeding, as defined by Saurin *et al*[12], were considered.

Statistical analysis

The Statistical Package for Social Sciences (SPSS) program version 10.0 (Chicago, Illinois) was used for statistical analysis. Continuous data with normal distribution were expressed as mean ± SE, while those without as median (range). Differences between groups were evaluated using χ^2 test for qualitative variables and Student's *t* test for quantitative variables following a normal distribution or the Mann-Whitney *U*-test for those who failed the normality test. A *P* value of less than 0.05 was considered statistically significant. Logistic regression analysis was used to find predictive variables of findings, which were identified in terms of odds ratio (OR) with 95% confidence intervals (95% CI).

Table 1 CRF patients' characteristics

No. of patients	17
Etiology of renal failure, *n* (%)	
Nephrosclerosis	4 (23.5)
Glomerulonephritis	3 (17.6)
Chronic pyelonephritis	1 (5.9)
Diabetic nephropathy	1 (5.9)
Other etiologies	3 (17.6)
Unknown	5 (29.4)
Duration of CRF (mean ± SE), mo	139.9 ± 13.0
Median serum creatinine (range), mg/dL	3.2 (2.0 - 9.4)
Serum urea (mean ± SE), mg/dL	133.9 ± 11.0

RESULTS

A total of 17 CRF patients fulfilled the inclusion criteria (7 predialysis patients, 4 on maintenance hemodialysis and 6 renal transplant recipients). CRF etiology and duration as well as biochemical parameters are shown in Table 1. Duration of dialysis ranged between 20 to 60 mo (mean ± SE: 38.7 ± 3.8) and the treatment schedule consisted of 4 h hemodialysis procedures three times weekly. Post-transplantation period ranged between 16 to 93 mo (mean ± SE: 53.2 ± 12.5). Immunosuppressive treatment in transplant recipients consisted of a combination of mycophenolate mofetil (MMF), cyclosporine and prednisone (3/6 patients), MMF, tacrolimus and prednisone (2/6) and MMF, sirolimus and prednisone (1/6). Two out of 17 CRF patients were taking low-dose aspirin and 1 non-steroidal anti-inflammatory drug (NSAID).

The group of non-CRF patients consisted of 51 patients with obscure bleeding. Table 2 shows the demographic and clinical characteristics of CRF and non-CRF patients. The two groups were found comparable regarding indications for WCE (overt, occult bleeding), NSAID or aspirin use, comorbid diseases, hemoglobin level and need for previous transfusions.

CRF patients completed the procedure uneventfully. One case of capsule retention was observed in a non-CRF patient with an adenocarcinoma of the mid ileum. In 3 (17.6%) CRF and 10 (19.6%) non-CRF patients, the capsule did not reach the colon, and, therefore, the entire SB was not imaged. Causes of failure of the capsule to reach the colon within the recording time in CRF patients were: slow gastric passage (1 case), presence of food

Table 2 Demographic and clinical characteristics of CRF and non-CRF patients

	CRF patients	Non-CRF patients	*P*
No. of patients	17	51	
Male/female	12/5	36/15	NS
Age (mean ± SE), yr	57.1 ± 2.5	57.2 ± 2.1	NS
Indication of WCE (overt/occult bleeding)	11/6	33/18	NS
NSAIDs or aspirin use, *n* (%)	3/17 (17.6%)	14/51 (27.5%)	NS
Comorbid diseases, *n* (%)	10/17 (58.8%)	23/51 (45.1%)	NS
Hb (mean ± SE), g/L	84.3 ± 2.0	87.7 ± 1.3	NS
Need for previous transfusions, *n* (%)	6/17 (35.3%)	28/51 (54.9%)	NS

NS: Non-significant.

Table 3 Small bowel findings revealed by wireless capsule endoscopy

SB findings	CRF patients *n* = 17 (%)	Non-CRF patients *n* = 51 (%)
Angiodysplasia	8 (47.0)	9 (17.6)
Single ulcer	2 (11.8)	3 (5.9)
Ulceration with cobblestoning and stricture	1 (5.9)	3 (5.9)
Non-specific ulcers, erosions, submucosal bleeding, erythema and edema	1 (5.9)	-
Fresh blood without an obvious explanation	-	2 (3.9)
Tumor	-	2 (3.9)
Multiple erosions	-	2 (3.9)
No findings	5 (29.4)	30 (58.8)

impairing the capsule progression (1 case), and no clear reason (1 case). Gastric emptying time in CRF patients ranged from 5 to 288 min (median: 20) and SB transit time from 99 to 393 min (median: 275).

WCE findings of SB in both CRF and non-CRF patients are listed in Table 3. In CRF patients, the most frequent type of SB lesion identified was angiodysplasia (8/17, 47%; two actively bleeding) (Figure 1). Patchy mucosal redness and a big jejunal ulcer (Figure 2) were revealed in 2 patients, 1 of which had a recent history of NSAIDs use. Ulceration, cobblestoning, stricture and a pattern of discontinuous involvement of the mid ileum, suggestive of Crohn's disease, were found in 1 patient. Finally, in 1 patient with erythema, edema, submucosal bleeding, erosions and non-specific ulcers of the distal ileum, caecum and ascending colon (Figure 3), tissue diagnosis of CMV enterocolitis was subsequently made by means of ileocolonoscopy and biopsies. Preceded colonoscopy in another institution, ten days earlier, had missed the lesions. Consequently, the diagnostic yield of WCE in CRF patients with obscure bleeding was significantly higher (70.6%, 12/17) compared with the non-CRF patients (41.2%, 21/51) ($\chi^2 = 4.42$, $P < 0.05$).

Angiodysplasia was the most frequent finding in the SB of CRF patients. A single lesion was found in 5 patients, 2 lesions in 2 and 3 in 1. Angiodysplasias were located in jejunum (5 patients), ileum (2 patients) and both (1 patient).

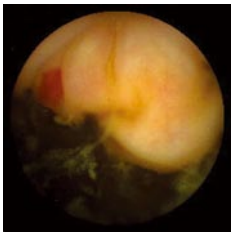

Figure 1 Single ileal angiodysplasia revealed by WCE.

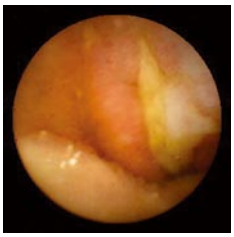

Figure 2 Patchy mucosal redness and a big jejunal ulcer detected by WCE.

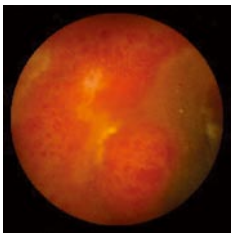

Figure 3 Wireless capsule endoscopy showing erythema, edema, submucosal bleeding and ulcer of the distal ileum in a renal transplant recipient.

We assessed presence of CRF, age, sex, comorbid diseases, hemoglobin level at the time of the procedure and need for previous transfusions as potentially relevant independent variables for angiodysplasia in both CRF and non-CRF patients. Univariate logistic regression revealed only CRF as a significant predictive factor for angiodysplasia (OR = 4.1, 95% CI = 1.3-13.7, $P < 0.05$). Moreover, we evaluated sex, age, duration of CRF and comorbid diseases as potentially relevant independent variables for SB angiodysplasias in CRF patients. SB angiodysplasias seem to be associated only with the duration of CRF (OR = 1.03, 95% CI = 0.99-1.06, $P < 0.1$).

In view of the WCE findings, 4/8 patients with angiodysplastic lesions within the reach of push enteroscopy were referred for endoscopic treatment with argon plasma coagulation and 1 patient with 3 angiodysplasias received estrogen therapy. Pharmacotherapy was provided in the patient with Crohn's disease as well as in the patient with CMV enterocolitis, and discontinuation of NSAIDs in the patient with the big jejunal ulcer was undertaken. Iron supplementation was decided to be the only measure in the remaining 3 cases with angiodysplasias and in the patient with the jejunal ulcer.

DISCUSSION

Gastrointestinal bleeding is a common complication of advanced CRF[1,2]. Although there have been many studies concerning implication of upper gastrointestinal tract and colon, data concerning SB are limited. WCE is a new method for the investigation of SB pathology, but already has an established role in cases of obscure gastrointestinal

bleeding[8]. We conducted this study in order to investigate SB pathology in CRF patients with obscure gastrointestinal bleeding and assess the diagnostic and therapeutic yield of the method in this group of patients. To our knowledge, there is only one case report on this issue[13].

According to the preliminary results of our ongoing study, angiodysplasia was recognized as a sole causative lesion of obscure bleeding in a markedly higher percentage, 47% in CRF patients *vs* 17.6% in non-CRF patients. A high prevalence (66.7%) of SB angiodysplasia among 6 CRF patients has also been reported in a recent study on the impact of WCE in obscure gastrointestinal bleeding[14]. In our study, logistic regression analysis showed that CRF was a significant predictive factor for SB angiodysplasia (OR = 4.1, 95% CI = 1.3-13.7, $P < 0.05$). Furthermore, our data suggested that the prevalence of angiodysplasia seemed to be inversely related with the duration of CRF, although this finding was statistically insignificant (OR = 1.03, 95% CI = 0.99-1.06, $P < 0.1$). Previous reports on the prevalence of angiodysplasia in CRF patients, as well as its association with patient's age and duration of the disease are conflicting[15-18]. However, all those studies used data obtained by conventional upper gastrointestinal endoscopy.

The diagnostic yield of WCE in CRF patients with obscure bleeding was significantly higher (70.6%) than that in non-CRF patients ($\chi^2 = 4.42$, $P < 0.05$), which is in agreement with previous data[19-21]. Given that WCE has already become the method of choice in the investigation of patients with obscure bleeding[8], its value in the subgroup of CRF patients is even more crucial.

WCE findings led to plan a specific intervention in 8/12 patients with positive findings. Therapeutic measures included push enteroscopy with coagulation of angiodysplasias in 4 patients, pharmacotherapy in 3 and discontinuation of medication in another 1.

In conclusion, according to the preliminary results of our ongoing study, SB angiodysplasia has an increased prevalence among CRF patients with obscure bleeding. WCE is useful in diagnosis of gastrointestinal pathologies and in planning appropriate therapeutic intervention and, therefore, should be included in the work-up of this group of patients.

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RAPID COMMUNICATION

Muscle fatigue in women with primary biliary cirrhosis: Spectral analysis of surface electromyography

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Abstract

AIM: To evaluate the myoelectric manifestations of peripheral fatigability in patients with primary biliary cirrhosis in comparison to healthy subjects.

METHODS: Sixteen women with primary biliary cirrhosis without comorbidity and 13 healthy women matched for age and body mass index (BMI) completed the self-reported questionnaire fatigue impact scale. All subjects underwent surface electromyography assessment of peripheral fatigability. Anterior tibial muscle isometric voluntary contraction was executed for 20 s at 80% of maximal voluntary isometric contraction. During the exercise electromyographic signal series were recorded and root mean square (expression of central drive) as well as mean and median of electromyographic signal frequency spectrum (estimates of muscle fatigability) were computed. Each subject executed the trial two times. EMG parameters were normalized, then linear regression was applied and slopes were calculated.

RESULTS: Seven patients were fatigued (median fatigue impact scale score: 38, range: 26-66) and 9 were not fatigued (median fatigue impact scale score: 7, range: 0-17). The maximal voluntary isometric contraction was similar in patients (82, 54-115 N) and controls (87, 74-101 N), and in patients with high (81, 54-115 N) and low fatigue impact scale scores (86, 65-106 N). Root mean square as well as mean and median of frequency spectrum slopes were compared with the Mann-Whitney U test, and no significant difference was found between fatigued and non-fatigued patients and controls.

CONCLUSION: No instrumental evidence of peripheral fatigability can be found in women with primary biliary cirrhosis but no comorbidity, suggesting that fatigue in such patients may be of central origin.

Key words: Primary biliary cirrhosis; Cholestasis; Surface electromyography; Peripheral fatigue; Muscle fatigue; Fatigability; Root mean square; Mean frequency; Median frequency; Fatigue impact scale

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INTRODUCTION

Fatigue affects 60%-80% of patients with primary biliary cirrhosis (PBC), and is considered to be specifically associated with PBC[1-3]. However, current understanding of fatigue pathophysiology in PBC is limited and comorbidity may play a significant role in fatigue pathophysiology[4].

The comprehension of this symptom has been hampered by the complexity of its nature and relationships with somatic illnesses, psychological disturbances, and stress reactions. Moreover, fatigue is subjective and difficult to measure[5]. The most used instruments for measuring fatigue in PBC are self-reported questionnaires such as the fatigue impact scale (FIS)[3,6-10].

Several studies support the hypothesis that fatigue has a central origin[10-13]. In contrast, Goldblatt and colleagues[7] assessed muscle fatigability in PBC patients with a grip strength protocol, and found that patients with high FIS scores are characterized by an accelerated decrease in muscle function compared with both healthy controls and non-fatigued patients.

However grip strength cannot distinguish if muscle fatigability is due to a central or a peripheral dysfunction, and it is not able to detect volitional components. Moreover, Stanca *et al*[14] have not found any difference in grip strength between patients with high FIS scores and controls. No study has completely excluded a peripheral component of fatigue in PBC patients.

A validated surface electromyographic technique which provides objective measures of peripheral fatigability

has been developed[15-17]. This method is able to monitor central drive and has been applied in both physiological and pathological conditions, but never in chronic liver diseases[18-20].

The aim of this study was to investigate if myoelectric manifestations of peripheral fatigability during voluntary isometric contractions in women with PBC, but no comorbidity, are significantly different from those in healthy subjects matched for their age, sex and body mass index (BMI).

MATERIALS AND METHODS

Patients

Fifty-seven patients with PBC were screened for several common diseases (renal failure, anaemia, electrolyte imbalance, thyroid-associated disease, rheumatic fibromyalgia, polyneuropathy and diabetes) for assumption of drugs that could cause fatigue (b-blockers, *etc.*).

A subgroup of 16 women without nonhepatic causes of fatigue underwent EMG assessment of peripheral fatigability. The other patients did not participate in the study because of nonhepatic causes of fatigue or because they denied their consent. All patients were stable with no history of bleeding, ascites or encephalopathy. Laboratory investigations included blood cell count, liver function tests, detection of creatinine, thyroid hormones, HbA1c, iron, serum electrolytes, and anti-mitochondrial antibodies. All patients were treated with ursodeoxycholic acid (UDCA) at the time of the study.

Controls

Thirteen healthy women comparable for age and BMI who declared that they did not assume drugs or suffer from any disease served as controls.

Ethics

The study protocol conformed to the ethical guidelines of the 1996 Helsinki Declaration and all subjects gave their informed written consent to participating in the experimental study.

Questionnaires

All subjects completed two self-reported questionnaires in a proper setting: fatigue impact scale and Rand MOS depression screening. FIS is a multidimensional scale composed of 40 items providing a global score (maximum 160), containing the intermixed physical, cognitive (maximum score 40 each) and psycho-social (maximum score 80) domains of fatigue[21]. FIS has been recently validated in PBC patients[22].

Rand MOS depression screening is composed of 8 items to screen the depressive disorders (major depression and dysthymia) and has been used in general and chronic disease populations, and in PBC patients[23]. It does not consider physical depressive symptoms that can be found also in abdominal diseases.

Experimental protocol

EMG analysis of isometric contractions of the dominant anterior tibial muscle was conducted in this study. This muscle was chosen because of its simple anatomy and because it has been analyzed by other investigators for fatigue studies[15].

Subjects laying on a bed were asked to put on a specially designed boot. The ankle joint was fixed with Velcro straps. A force transducer (Digitalanzeiger mod. 9180) was displayed to measure the force produced by the dorsal extension of the feet, and a display located at bed side showed the instantaneous force values expressed in Newton.

Before EMG signal acquisition, the subjects were requested to perform a short (5 s) maximal voluntary isometric contraction three times with a 2 min rest between each contraction. The mean maximal voluntary isometric contraction (MVC) of the three recorded maximal force values was used as the reference value.

Fatigue test for anterior tibial muscle isometric contraction was performed for 20 s at 80% of MVC. This target force was reached and held by means of the visual feedback display located at bed side showing the instantaneous force values. Standardised verbal encouragement was given throughout this force-failure point. Fatigue test was performed two times with 5 min rest between the two sessions.

The following clinical force parameters were considered: MVC and fatigue threshold (FT) which is time necessary to reduce target force more than 10%. The first one could be considered as an estimation of the muscle force, while the second parameter could be considered as the clinical evidence of fatigability.

The skin was cleaned and two pregelled disposable surface electrodes (Bionen Firenze) were placed 20 mm apart on the longitudinal midline of the dominant anterior tibial muscle between the motor point and the tendon. A ground electrode was attached to the knee. EMG signals were recorded during each session of the fatigue test.

Neurophysiologists were blinded to FIS scores of the subjects.

Analysis of data

Twenty series of 500 ms windows were taken from the 20 s data of the EMG signal of the fatigue test. For each window, root mean square (RMS) amplitude of the EMG signal and spectral analysis (512 points, Hanning window processing, fast Fourier transform) were applied. The mean MNF and median MDF values of EMG signal frequency spectrum were computed. EMG parameters were normalized to the values generated at the beginning of each session. Then, linear regression was applied to the time-series of RMS, MNF and MDF to estimate their rate of change (slope of their respective linear regression).

MNF and MDF slopes are an estimate of muscle fatigability[15]. MNF and MDF tended to decrease during the trial in normal subjects, thus the slope values of their respective linear regression were negative. Subjects with a higher fatigability have greater absolute values of MNF and MDF slopes. RMS is an expression of the central drive and its value tended to increase during the trial, thus the slope value of its linear regression was positive. The analysis of peripheral fatigability was possible only when RMS slope was positive. The finding of a low central drive

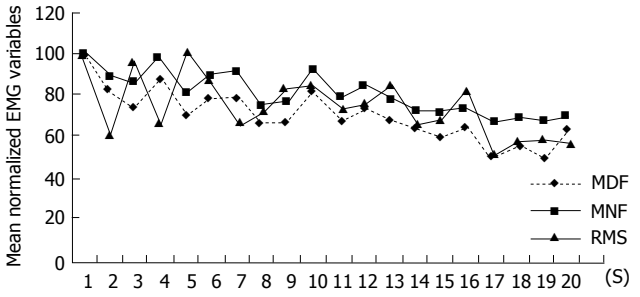

Figure 1 Normalized values of EMG variables during voluntary isometric contractions at 80% MVC from a patient with low central drive. In this case the trial was not included in the analysis of peripheral fatigue.

could be used as an index of the central pathology or it could be due to a volitional component.

Statistical analysis

The Mann-Whitney *U* test (non-parametric test for independent samples) was used to identify whether significant differences in MVC existed between subject groups. Statistical comparison was made between controls and patients, between controls and subgroups of fatigued and non-fatigued patients.

EMG parameters were analyzed during each recording session by repeated measurements (ANOVA). The univariate solution was obtained after correction for the Greenhouse-Geisser factor when appropriate, to protect against type I errors associated with nonsphericity of data. In each case, the approximate F value associated with the univariate test was reported. Variables showing significant effects or interactions ($P < 0.05$) were subjected to *post hoc* testing (Scheffé) using an alpha level of less than 0.05. Sample regression of EMG parameters was used to evaluate the inter-observation variation between the first and second session.

All statistical analyses were performed using StatView for Windows.

RESULTS

Clinical features of the 16 selected patients (median age: 54 years, range: 37-73 years; median BMI: 23 kg/m^2, range: 17.9-28.5 kg/m^2) and the controls (median age: 51 years, range: 45-64 years; median BMI: 23.2 kg/m^2, range: 18.2-28.4 kg/m^2) were comparable. Our PBC population was composed of 2 patients in stage I, 6 in stage II, 7 in stage III and 1 in stage IV. Liver biopsies were taken no more than 5 years of post-muscle testing, during which time clinical and laboratory features were not substantially modified.

We divided the patients into two groups according to their FIS scores: seven fatigued (median total FIS: 38, range: 26-66) and nine non-fatigued (low or very low total FIS; median total FIS: 7, range: 0-17). Depression assessed by Rand MOS Depressions Screening was present in two patients.

The MVC force was not statistically different between patients (median: 82 N; range: 54-115 N) and controls (median: 87 N; range: 74-101 N). No difference was found also between patients with high (median: 81 N; range: 54-115 N) and low FIS scores (median: 86 N; range: 65-106 N). Fatigue threshold was reached only in two patients.

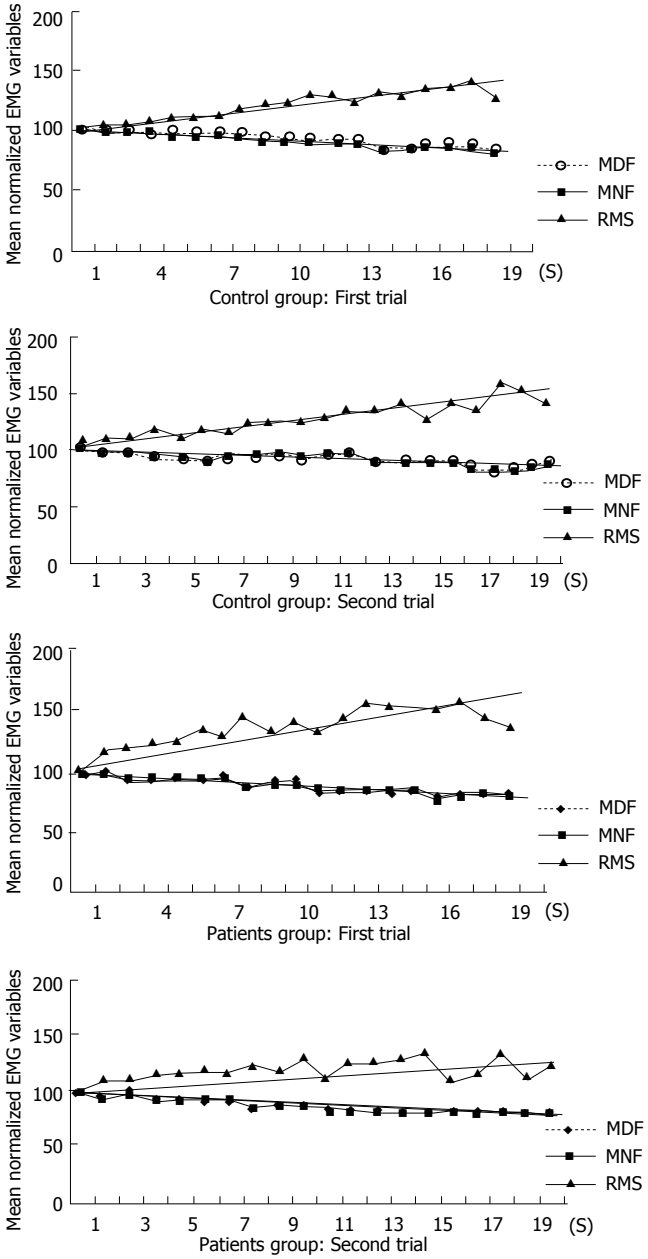

Figure 2 Mean normalized values of EMG variables during voluntary isometric contractions at 80% MVC from patients and controls. The time course of MNF, MDF, RMS and force were combined to produce a "fatigue plot". Plots were obtained by normalizing each variable with respect to the initial value of its own regression line.

RMS did not increase during the trial in two patients (Figure 1), so EMG analysis of peripheral fatigability was performed on the remaining 14 patients. These two patients reached the fatigue threshold. Fatigue testing was performed twice in each patient, consequently 28 slope values of each parameter for patients and 26 for controls were obtained.

The mean normalized values of all variables (MNF, MDF, RMS) during voluntary isometric contractions at 80% MVC from patients and controls are shown in Figure 2. RMS ($P = 0.84$), MNF ($P = 0.66$) and MDF ($P = 0.4$) slopes were not significantly different between fatigued

and non-fatigued patients and controls, as well as between the two groups of patients. Inter-observation variation between the first and second sessions was as follows: $r = 0.88$, $P < 0.01$ for MDF; $r = 0.93$, $P < 0.01$ for MNF; $r = 0.80$, $P < 0.05$ for RMS.

DISCUSSION

Fatigue is an overwhelming sense of tiredness, lack of energy or feeling of exhaustion. It is frequently present even at rest. Fatigue is a multidimensional symptom caused by the interaction of physical, psychosocial and cognitive factors. It cannot be confused with weakness or fatigability. The last one in particular is a generalised sense of exhaustion, not present at rest, affecting the patient after a few minutes of physical activity and disappears after a short rest[24].

Fatigue muscle arises only when muscle fibres are sufficiently activated and consequently develop an effective contraction. Stimulation can be obtained artificially with electrodes or through the physiologic voluntary nervous pathways. The first method gives a more constant elicitation, however it is not physiologic and it is quite an irritation for patients. The second one employs the will of the subjects in keeping the contraction of the analyzed muscle. In our study, isometric contraction of the dominant anterior tibial muscle was voluntary.

Fatigue can be evaluated using a particular EMG analysis. In fact, when muscle fibres contract, they generate electric activities which are recordable with EMG electrodes. EMG signals have different amplitudes and frequencies. The sum of all signals generated by all muscle fibres during the contraction provides a frequency spectrum. As the contraction carries on and the fatigue arises, the frequency spectrum changes. In particular, the mean and median frequency spectrum values (MNF and MDF) decrease proportionally with the duration of contraction and the fatigue of muscle.

As fatigue increases, the subjects effort in keeping a target force increases as well. This effort corresponds to an increase of the stimulation of muscle fibres, in other words we have a higher central drive. The increment of central drive determines the increase of amplitude of the frequency spectrum. Thus EMG analysis of the frequency spectrum can evaluate also the central drive of the subjects during the isometric contraction. Root mean square is an estimate of the amplitude of frequency spectrum, and its value tends to increase during exercise. In our study, as in others, this parameter was used as an estimate of central drive[15].

When a subject develops fatigue and is not able to keep a target force, it can be due to an inadequate force generation by muscle fibres or by a lack of central drive (peripheral and central fatigue)[25]. If muscle fibres are not sufficiently stimulated because of a lack of central drive, they do not contract enough to develop fatigue, thus in this condition peripheral fatigue cannot be evaluated.

Central drive is not sufficient for two main reasons: the subject is not motivated and does not make enough effort to execute an adequate muscle contraction and the subject is affected by a pathologic neurological central condition that does not allow activation of the muscle. Hence peripheral fatigue can be analyzed only in the presence of an adequate central drive, and central drive can be low for a lack of motivation or for a neurological pathology.

To the best of our knowledge, this is the first study in patients affected by PBC in which it has been used as an EMG technique able to quantify fatigability and analyse the presence of peripheral dysfunction.

In our population MVC values were similar in patients and controls. These findings agree with those of Goldblatt *et al*[7] and we can suppose that PBC patients are not weaker than the general population. In addition, fatigue testing showed that fatigability in women affected by PBC without comorbidity was not significantly different from that in healthy controls matched for age, sex and BMI. Moreover, we did not find any significant difference in peripheral fatigability between fatigued and non-fatigued patients as assessed by FIS.

Two patients had a defective central drive as shown by a flat RMS slope and both of them had high FIS scores and were not able to maintain the 80% MVC target (Figure 1). We do not know if this lack of central activation was due to a volitional component or to a central dysfunction. In fact, our EMG technique was not able to analyze central dysfunction. These two patients had a normal MVC and standard EMG did not show any pathological neuromuscular abnormality. Interestingly, both women were positive for Rand MOS Depression Screening. So, according to the previous studies showing a positive correlation between depression and fatigue, we may suppose a lack of motivation in these two patients[2,3,6]. If they would have been analysed only with force recording, as done in previous studies, they would be classified as subjects with high muscle fatigability[7].

In conclusion, no evidence of peripheral fatigability in PBC patients without comorbidity is demonstrable. If these data will be confirmed, fatigue pathogenesis in PBC patients may be related to a central mechanism. This central alteration may be due to a common genesis with the liver disease, or in alternative to one or more causes not related to the hepatic damage (specific or not specific symptom of PBC). Some data support the last hypothesis, in fact two recent reports have failed to demonstrate a significant difference in FIS scores between patients and controls and no correlation has never been found between fatigue and parameters of disease severity and activity[2,4,6,9,14]. Future research should be addressed on the investigation of central fatigue and the role of depression, which is the only parameter significantly associated with fatigue in several studies[2,3,6].

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RAPID COMMUNICATION

Microheterogeneity of acute phase proteins in patients with ulcerative colitis

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Abstract

AIM: To estimate the serum α_1-antichymotrypsin (ACT), α_1-acid glycoprotein (AGP) and transferrin (Tf) concentrations and to evaluate the microheterogeneity of these acute phase proteins in patients with ulcerative colitis.

METHODS: Twenty-seven patients with ulcerative colitis (UC) and 17 healthy control subjects were studied. The patients were categorised as severe (n = 9), moderate (n = 10) and mild groups (n = 8) using Truelove and Witts' classification of ulcerative colitis. Microheterogeneity of ACT, AGP and Tf was analysed by crossed immunoaffinity electrophoresis (CIAE) with concanavalin A. In all serum samples standard electrophoresis of serum proteins was performed, iron (Fe) concentration, total iron binding capacity (TIBC) and C-reactive protein (CRP) were also measured.

RESULTS: Our patients suffering from ulcerative colitis had significantly higher serum ACT and AGP concentrations and lower serum transferrin concentration in comparison to healthy subjects. Changes in concentrations of acute phase proteins were dependent on the activity of the inflammatory process. The glycosylation patterns of transferrin were related to the inflammation status. We also observed the correlation between ACT and AGP concentrations, patterns of transferrin glycosylation and changes in standard protein electrophoresis or blood cell count.

CONCLUSION: The glycosylation patterns of transferrin obtained from patients suffering from ulcerative colitis are highly branched and sialylated compared with those obtained from healthy subjects. In contrast, the glycosylation patterns of transferrin do not differ according to the activity index of ulcerative colitis. The microheterogeneity patterns of AGP and ACT are similar in ulcerative colitis patients and healthy subjects.

Key words: Glycoproteins; Acute phase response; Ulcerative colitis

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INTRODUCTION

In ulcerative colitis, a chronic inflammatory disease of unknown etiology, proinflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6 and IL-2, play a major pathophysiological role[1-5]. Acute phase response is a systemic answer to mechanical injuries, infections as well as an unspecific inflammation. Acute phase proteins (APP) play an important role in this non-specific immune reaction. These proteins are produced by the liver in answer to cytokine mediation. The concentrations of many APP have been studied in ulcerative colitis patients[6-8]. Measurement of APP serum concentrations offers a valuable means of assessing the intensity of inflammatory bowel disease. Unsurprisingly, various studies have shown the correlation between these inflammatory mediators and acute phase proteins, such as C-reactive protein (CRP), α_1-acid glycoprotein (AGP), α_1-antichymotrypsin (ACT) and serum amyloid A (SAA)[9-11]. A acute phase proteins and clinical signs are also of a high value for the treatment and remission of ulcerative colitis. However, the etiological and clinical importance of these proteins is still not completely revealed.

Except for CRP, albumin and SAA, all acute phase proteins are glycoprotiens. Glycosylation is the primary cause of microheterogeneity in proteins. It is an enzyme-directed and site-specific process. Protein sugar prints are conserved, not random and determined independently from the synthetic rate of protein. There is O-linked glycosylation to the hydroxy oxygen of serine and threonine side chains and N-linked glycosylation to the amide nitrogen of asparagine side chains. The N-glycan chains of α_1-AGP, α_1- ACT and Tf differ in their branching, showing

Table 1 Clinical characteristics of patients with UC (mean ± SD)

Trulove and Witts' classification	UC (n = 27)	Severe (n = 9)	Moderate (n = 10)	Mild (n = 8)	P
Female (n)	16	4	6	6	
Male (n)	11	5	4	2	
Age (yr)	41.80 ± 18.00	40.20 ± 20.20	38.00 ± 16.00	48.40 ± 18.30	NS
Hemoglobin (g/dL)	12.01 ± 2.21	11.17 ± 1.92	11.88 ± 2.14	13.11 ± 2.38	< 0.05
PLT (thsd/mm^3)	352.00 ± 190.62	428.78 ± 116.65	358.90 ± 235.76	257.00 ± 173.18	< 0.05
Fe (μg/dL)	73.90 ± 35.60	52.33 ± 19.20	73.20 ± 26.71	99.00 ± 45.19	< 0.05
WBC (thsd/mm^3)	8.68 ± 3.99	10.20 ± 5.61	9.03 ± 3.13	6.58 ± 1.52	< 0.05
Total protein (g/dL)	6.15 ± 0.99	5.72 ± 1.20	6.52 ± 0.71	6.20 ± 0.93	< 0.05
Albumin (g/dL)	3.22 ± 0.82	2.70 ± 0.78	3.63 ± 0.59	3.40 ± 0.89	< 0.05

bi-, tri-, and tetra-antennary structures[12-14]. These serum N-glycoproteins physiologically occur in few variants, called microheterogeneity. During acute phase response both concentrations and percentage of individual variants are changed.

Glycosylation may play a role in cell-cell adhesion. The highly branched and sialylated form of AGP which is the ligand for cell adhesion molecules such as E-selectin and P-selectin, inhibits migration of neutrophils, monocytes and T-cells, and ameliorates complement activity[15]. The asialylated carbohydrate-deficient variant of AGP appears mainly in sera of patients after acute inflammation, infection, burn or other severe tissue damage or necrosis[16]. The immunomodulatory properties of AGP have been shown to depend on its glycosylation. The inhibition of lymphocyte proliferation depends on the branching degree of the glycans of AGP[17]. The sialylated acute phase proteins protect against immune complex-induced injury[18]. An increase of sialyl-variants of APP seems to be a mechanism responsible for feedback inhibition of leucocyte migration to inflamed tissues. The heterogeneity of the glycosylation pattern of AGP that has been found in patients with ulcerative colitis reflects changes in response to inflammation[19].

The changes in patterns of glycosylation of transferrin have been observed in iron deficiency anaemia, rheumatoid arthritis, liver cirrhosis or in physiological status such as pregnancy[20]. The microheterogeneity pattern of Tf shifts under inflammatory conditions towards highly branched glycans. Differences in glycosylation of Tf seem to alter the iron metabolism.

The aim of this study was to estimate the changes in microheterogeneity in glycosylation patterns of α_1-AGP, α_1-ACT and Tf in sera of patients suffering from ulcerative colitis.

MATERIALS AND METHODS

Twenty-seven patients suffering from biopsy-proven ulcerative colitis and 17 healthy control subjects were studied.

The sera were separated by centrifugation after clotting and stored at -70℃. Rocket immunoelectrophoresis was used to determine the total serum concentrations of AGP, ACT, Tf, and CRP.

Microheterogeneity of ACT, AGP and Tf was analysed by crossed immunoaffinity electrophoresis (CIAE) with lectin-concanavalin A. The lectin-concanavalin A was included in the first-dimension gel as the diantennary-specific affinocomponent. Separation of different glycoforms of proteins in sera was done *via* electrophoresis of these fluids with a concanavalin A-containing (Con A) polyacrylamide slab gel. Proteins lacking glycans of the diantennary type are not retarded by Con A, whereas proteins containing one or more diantennary glycans bind to Con A, and are electrophoretically retarded in the gel. Detection of the separated glycoforms was achieved through electrophoresis in the second dimension, using the polyclonal protein-specific anti-IgG antibody. The resulting precipitation lines were stained, and the relative occurrences of lectin-retarded and lectin-nonretarded glycoforms were calculated from the areas under the curves.

AGP, ACT and Tf in sera were separated into four variants: W 0-3 for AGP, A 1-4 for ACT, T 1-4 for Tf. Furthermore, the following parameters were measured: standard electrophoresis of serum proteins, iron (Fe) concentration, total iron binding capacity (TIBC), CRP, haemoglobin concentration and blood cell count.

RESULTS

Clinical characteristics and laboratory data

Twenty-seven patients suffering from ulcerative colitis were studied (16 females, 11 males). The patients were categorised according to the clinical activity index for ulcerative colitis using Truelove and Witts' classification of ulcerative colitis (severe n = 9, moderate n = 10 and mild n = 8). There were no significant differences in age, gender or body mass index (BMI) among the three studied groups (mean age 41.8 ± 18 years, mean BIM 21.858 ± 3.58 kg/m^2). The nutritional status evaluated by physical characteristics was also comparable in the three groups (Table 1).

The haemoglobin concentration and platelet count were significantly lower in the severe group than in the mild group (11.17 ± 1.92 g/dL and 13.11 ± 2.38 g/dL, respectively, $P < 0.05$; and 428.78 ± 116.65 thsd/mm^3 and 257 ± 173.18 thsd/mm^3, respectively, $P < 0.05$). The serum iron concentration was significantly lower in the severe group than in the moderate (52.3 ± 19.2 μg/dL and 73.3 ± 26.7 μg/dL, respectively, $P < 0.05$) and mild groups (52.3 ± 19.2 μg/dL and 99 ± 45.2 μg/dL , respectively, $P < 0.05$). The white blood cell count was higher in the severe group than in the mild group (10.2 ± 5.61 thsd/mm^3 and 6.48 ± 1.6 thsd/mm^3, respectively, $P < 0.05$).

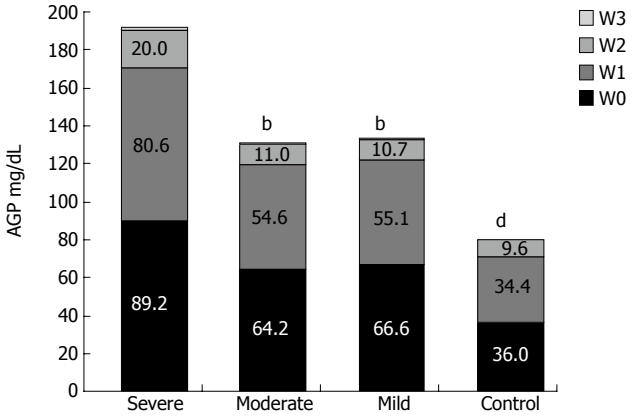

Figure 1 Microheterogeneity of AGP in sera of UC patients and healthy controls. $^{b}P < 0.001$ *vs* severe UC patients; $^{d}P < 0.001$ *vs* healthy subjects.

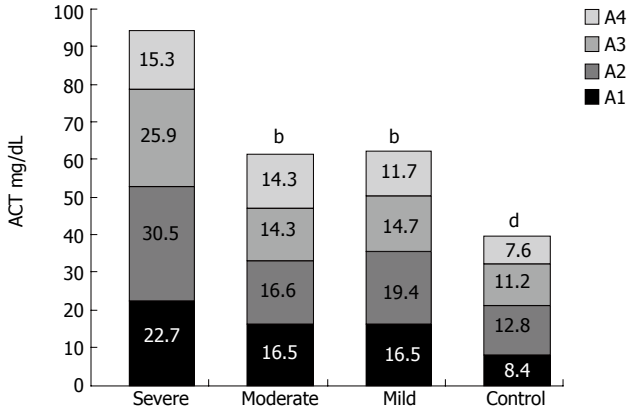

Figure 2 Microheterogeneity of ACP in sera of UC patients and healthy controls. $^{b}P < 0.001$ *vs* severe UC patients; $^{d}P < 0.001$ *vs* healthy subjects.

There were also significant differences in total protein concentration and albumin concentration between severe and moderate groups (5.72 ± 1.2 g/dL and 6.52 ± 0.71 g/dL, respectively, $P < 0.05$; 2.7 ± 0.78 g/dL and 3.63 ± 0.59 g/dL, respectively, $P < 0.05$).

Acute phase proteins

The CRP concentration was significantly higher in the severe group than in the moderate and mild groups (49 ± 34 mg/L and 5 ± 8.4 mg/L, respectively, $P < 0.001$; 49 ± 34 mg/L and 15 ± 24.42 mg/L, respectively, $P < 0.05$).

The serum concentration of AGP was significantly higher in all UC patients than in healthy subjects (151.3 ± 65.5 mg/dL and 80 ± 11 mg/dL, respectively, $P < 0.001$). However, there were also significant differences between the three groups. The highest concentration of AGP was found in the severe group (severe to moderate group: 191.1 ± 77.6 mg/dL and 130.3 ± 40.7 mg/dL, respectively, $P = 0.02$; severe to mild group: 191.1± 77.6 mg/dL and 132.9 ± 62.4 mg/dL, respectively, $P = 0.053$) (Figure 1).

The serum concentration of ACT was also elevated in all studied patients compared to that of the control group (72.8 ± 29.5 mg/dL and 40 ± 5.2 mg/dL, respectively, $P < 0.001$). Similar to AGP, the ACT concentration was significantly higher in the severe group than in the moderate and mild groups (severe to moderate group: 94.3 ± 36.7 mg/dL and 61.8 ± 18 mg/dL, respectively, $P = 0.01$; severe to mild group: 94.3 ± 36.7 mg/dL and 62.2 ± 19.2 mg/dL, respectively, $P = 0.02$) (Figure 2). There were no statistically significant differences in AGP and ACT serum concentrations between the moderate and mild groups.

As a negative acute phase protein, serum transferrin concentration was decreased in patients with ulcerative colitis compared to that in the healthy volunteers (237.0 ± 82.3 mg/dL and 352.0 ± 59.1 mg/dL, respectively, $P < 0.001$). There was no difference in the serum transferrin concentration between the three groups (Figure 3).

Microheterogeneity of acute phase proteins

The increased concentrations of AGP and ACT were not associated with a large shift in the microheterogeneity patterns.

Interestingly, we noticed a strong positive correlation

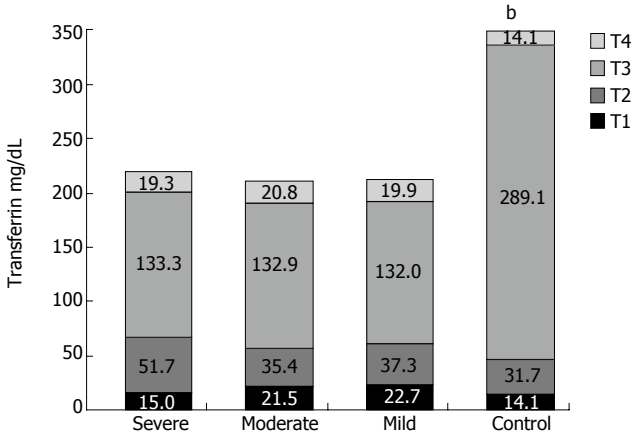

Figure 3 Microheterogeneity of Tf in sera of UC patients and healthy controls. $^{b}P < 0.001$ *vs* healthy subjects.

between the serum AGP concentration, particularly W1 glycoform of AGP and platelet count ($r = 0.578$, $P = 0.0016$). The negative correlation was found between the albumin and serum AGP concentrations ($r = -0.448$, $P < 0.05$) as well as ACT concentration ($r = 0.429$, $P < 0.05$). A particular strong negative relationship occurred between W1 serum fraction of AGP ($r = 0.444$, $P = 0.0296$) as well as A2 serum fraction of ACT and albumin concentration ($r = 0.578$, $P = 0.003$). There was also a positive correlation between the platelet count and the serum concentration of A2 glycoform of ACT ($r = 0.39$, $P < 0.05$).

Decreased serum transferrin concentration in ulcerative colitis patients was accompanied with redirection of glycan synthesis to the highly branched and sialylated glycan. We noticed that the percentage of T2 (bi-, tri- and tetra-antennary) and T1 (tetra- and more antennary) fractions of transferrin were significantly higher in the investigated groups than in healthy subjects, while the percentage of T3 (biantennary) and T4 (asialylated, carbohydrate-deficient) fractions were lower.

We noticed that the correlation between T2 variant of transferrin and platelet count, as well as $\alpha 2$ protein electrophoretic fraction was positive ($r = 0.432$, $P < 0.05$ and $r = 0.407$, $P < 0.05$). The negative correlation was found between this particular T2 variant and albumin

concentration ($r = 0.407$, $P < 0.05$).

The positive correlation between the serum concentration of T4 glycoform of transferrin and haemoglobin concentration was observed ($r = 0.405$, $P < 0.05$). We also found a negative correlation between T3 glycoform and AGP concentrations, as well as the α1 electrophoretic fraction of proteins ($r = 0.48$, $P < 0.05$ and $r = 0.475$, $P < 0.05$).

DISCUSSION

Measurement of acute phase proteins may bring new insights into the mechanisms of inflammatory reactions occurring in ulcerative colitis. In our study, both during remission and exacerbation of ulcerative colitis the glycosylation profiles of AGP and ACT were altered. AGP variant W3 was not present in sera of both healthy individuals and ulcerative colitis patients during remission. However, it appeared in sera of the same patients during exacerbation. This variant is always present in the sera of patients after inflammation, infection, burn or other severe tissue damage. Its presence in the sera of our studied patients was probably caused by the active necrotizing and inflammatory processes in the gut. The high concentration of ACT demonstrated in our patients, is also characteristic for disorders where tissue necrosis occurs (e.g. in burns).

Though the clinical image can confirm ulcerative colitis (UC) is a chronic inflammatory disease, alternations in APP concentrations and microheterogeneity depict rather a domination of "acute inflammatory image" that is probably caused by IL-6. Even in remission of UC the glycosylation profile of the best described AGP does not return to normal but remains altered, with a relative increase of Con A reactive (thus: biantennary) glycoforms. This constant inflammatory stimulation probably contributes to altered Tf glycosylation and, as a consequence to deteriorated iron metabolism and anaemia. In our study, the concentration of transferrin, a negative acute phase protein, was markedly decreased in the acute phase of ulcerative colitis, suggesting that low concentration and especially changes of the glycosylation profile of Tf may be responsible for the impaired iron metabolism in patients suffering from ulcerative colitis.

In sera of healthy individuals, transferrin separates into four variants: T1 weakly reacting with Con A (with mainly triantennary glycans), T2 (with one biantennary glycan and one triantennary glycan), T3 (with two biantennary glycans) and T4 (with probably defective sugars). The variant T3 covers about 80% of the total protein and is the main fraction able to bind to iron, consisting mainly of apotransferrin. Our study showed the alterations of Tf glycosylation in sera of patients suffering from ulcerative colitis. It is well known that existing anaemia in UC patients is not only to the blood loss with a stool, but also due to the chronic inflammatory process. It is possible that the different glycoforms of Tf have a different iron affinity that may affect the iron balance in the organism.

A correlation was shown between the concentration of transferrin T2 variant, bearing partially triantennary glycans and platelet count as well as α-2 protein electrophoretic fraction, whereas a negative correlation was shown between T2 variant and albumin concentration. We are thus able to show that inflammation influences the microheterogeneity of transferrin. The higher intensity of the inflammatory process is combined with deteriorated iron transport ability to transferrin, which may intensify anaemia.

The present study revealed a strong negative correlation between variant T1 of transferrin, α_1-acid glycoprotein and α_1-antichymotrypsin concentration. A negative correlation was observed between the patient status and iron concentration. Disturbances in the iron balance (low TIBC and iron concentration) reflected a higher amount of variant T1 of transferrin in the sera of UC patients.

In conclusion, glycosylation patterns of transferrin in ulcerative colitis patients shift to the highly branched and sialylated glycans compared with those in healthy subjects. However, the glycosylation patterns of transferrin do not differ according to the activity index of ulcerative colitis. The microheterogeneity patterns of AGP and ACT are similar in ulcerative colitis patients and healthy subjects, though the total concentrations of these acute phase proteins are increased due to the disease.

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RAPID COMMUNICATION

Smoking is not associated with nonalcoholic fatty liver disease

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Abstract

AIM: To analyze the relationship between smoking and nonalcoholic fatty liver disease (NAFLD).

METHODS: This is a cross-sectional study of a healthy population, carried out in a check-up unit of a university hospital in Mexico City. We enrolled 933 subjects, 368 current smokers (cases) and 565 persons who had never smoked (controls). Demographic, metabolic and biochemical variables were measured in the two groups. NAFLD was determined by ultrasound and metabolic syndrome according to ATPIII.

RESULTS: A total of 548 men (205 cases and 343 controls) and 337 women (114 cases and 223 controls) were included in the analysis. Statistical differences between cases and controls were observed only in high blood pressure prevalence (6.6% *vs* 11.3%, $P < 0.05$; cases and controls respectively), high-density lipoproteins (1.00 ± 0.26 *vs* 1.06 ± 0.28 mmol/L, $P < 0.005$), triglycerides (2.18 ± 1.49 *vs* 1.84 ± 1.1 mmol/L, $P < 0.001$), and erythrocyte sedimentation rate (11.3 ± 9.3 *vs* 13.5 ± 11.9 mm/h, $P < 0.001$). No differences were observed in the prevalence of NAFLD (22.27% *vs* 29.68%, P = NS) and metabolic syndrome (41.69% *vs* 36.74%, P = NS). Univariate analysis showed that smoking was not a risk factor for NAFLD (OR = 0.89, 95% CI 0.65-1.21).

CONCLUSION: No differences in NAFLD prevalence were observed between current smokers and nonsmokers, and furthermore, no differences were observed in heavy smokers (more than 20 packs/year), indicating that there is no relationship between smoking and NAFLD.

Key words: Smoking; Nonalcoholic fatty liver disease; Metabolic syndrome

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is an increasingly recognized condition that may progress to end-stage liver disease[1]. The clinical implications of NAFLD are derived mainly from its common occurrence among the general population (from 2.8% to 25%) and its potential to progress to fibrosis, cirrhosis and liver failure[2].

Obesity or a high body mass index (BMI) is a major risk factor for development of liver disease, and the prevalence of NAFLD is elevated 4.6-fold among obese people. In obese subjects, metabolic syndrome that links type 2 diabetes mellitus, dyslipidemia and hypertension to a visceral or male pattern of adipose tissue distribution is a more important risk factor for most comorbidities of obesity than obesity *per se*[3]. Other risk factors associated with NAFLD are waist circumference (> 102 cm for males and > 88 cm for females)[4], hyperinsulinemia, hypertriglyceridemia and impaired glucose tolerance or type 2 diabetes[5,6]. In fact, hepatic steatosis (HS) is now considered to be the hepatic manifestation of insulin resistance (IR)[7].

Smoking is a well-studied risk factor for many malignant neoplasms, cardiovascular diseases, chronic obstructive pulmonary diseases and other important diseases[8]. However, it was recently shown that smoking is associated with many of the risk factors for NAFLD, particularly obesity[9], IR, diabetes and dyslipidemia[10].

Basic and clinical research demonstrates that smoking alters enzymatic and inflammatory pathways in liver physiology[11,12], and is considered to be a risk factor for liver neoplasm, and affects the prognosis of chronic liver diseases[13,14].

Currently, there is no information available about the epidemiologic importance of smoking in NAFLD. The aim of this study was thus to investigate the relationship between smoking and NAFLD.

MATERIALS AND METHODS

Study population

We conducted a cross-sectional study in the Check-up

Unit of the Diagnostic Clinic at the Medica Sur Clinic and Foundation (a university hospital with subspecialty care) in Mexico City, Mexico. This hospital provides private care for mainly middle- and high-income individuals from Mexico City and the surrounding metropolitan areas. Our sample population was formed from a series of consecutive asymptomatic subjects who were referred to the Check-up Unit by their companies as an annual requirement. The study was approved by the Human Subjects Committee of The Medica Sur Clinic and Foundation as conforming to the ethical guidelines of the 1975 Declaration of Helsinki, and written informed consent was obtained from all participants before entry. After informed consent was obtained, all patients were asked to complete a questionnaire that included demographic and medical variables. Cases were defined as current tobacco smokers (and were classified according to the number of packets smoked per year), while controls were defined as patients who had never smoked.

Questionnaire

Subjects were asked to complete a questionnaire that asked for information on demographic data, age, gender, alcohol consumption, smoking habits, diabetes mellitus, hypertension, chronic liver disease, hyperlipidemia, and the use of drugs that predispose to NAFLD.

Physical examination

Body weight was measured, in light clothing and without shoes, to the nearest 0.10 kg. Height was measured to the nearest 0.5 cm. BMI was calculated as weight (kg) divided by height (m) squared. Waist circumference (to the nearest 0.1 cm) was measured at the midpoint between the lower border of the rib cage and the iliac crest, and hip circumference was similarly obtained at the widest point between hip and buttock.

Three blood pressure readings were obtained at 1 min intervals, and the second and third systolic and diastolic pressure readings were averaged and used in the analyses.

Smoking exposure

In a detailed questionnaire, all participants were asked about smoking frequency, duration and type of cigarette. Secondhand smoking was not evaluated. Subjects exposed to chemical or industrial gases were excluded, as were those regularly exposed to other environmental smoke (i.e., open fires using firewood and crop residues as fuel). Based on the number of cigarettes and years of smoking, we calculated pack/year (each pack was considered consisting of 200 cigarettes).

Metabolic syndrome

Participants having three or more of the following criteria were defined as having the metabolic syndrome. The criteria were defined according to the Executive Summary of the Third Report of the National Cholesterol Education Program[15]. (1) Abdominal obesity: Waist circumference > 102 cm in men and > 88 cm in women; (2) Hypertriglyceridemia: Triglycerides $\geqslant$ 1500 mg/L (1.69 mmol/L); (3) Low high-density lipoprotein (HDL) cholesterol: HDL < 400 mg/L (1.04 mmol/L) in men and < 500 mg/L (1.29 mmol/L) in women; (4) High blood pressure: $\geqslant$ 130/85 mmHg (17.33/11.33 kPa); (5) High fasting glucose: $\geqslant$ 1100 mg/L ($\geqslant$ 6.1 mmol/L).

Hepatic steatosis

The diagnosis of NAFLD was first suspected in those subjects with a record of at least six months of abnormal serum aminotransferase levels that were not related to other causes of liver disease, including hepatitis B and C virus infections, autoimmune disorders, alcohol consumption or hemochromatosis, and who had ultrasonographic findings compatible with HS. Real-time ultrasonographic studies were performed while the subjects were fasting. A 3.5 MHz transducer (Elegra; Siemens Medical Systems, Mountain Grove, CA, USA) was used to obtain the following images: sagittal view of the right lobe of the liver and right kidney; transverse view of the left lateral segment of the liver and spleen; transverse view of the liver and pancreas, and any focal areas of altered echotexture. The protocol used to evaluate the pattern of HS by ultrasound was graded according to the method of Saadeh *et al*[16]. In the second evaluation, all studies for each subject were viewed side-by-side in a masked fashion ($\varkappa$ = 0.92).

Analytical procedures

Plasma glucose of subjects in the fasting state was measured in duplicate using an automated analyzer. The coefficient of variation for a single determination was 1.5%. Cholesterol, HDL-cholesterol and triglyceride concentrations were measured by enzymatic colorimetric methods, using CHOL, HDL-C plus (second generation) and triglyceride assays (Roche Diagnostics Co., Indianapolis, IN, USA), respectively. Low-density lipoprotein (LDL) cholesterol concentrations were calculated using the Friedewald formula[17].

Statistical analyses

Means and standard deviations were used to describe the distributions of continuous variables in comparisons between smokers and controls. The nonparametric Mann-Whitney *U* test was applied because of the nonnormal distribution of some of these variables. By means of cross-tabulations, the risks associated with the probability of having HS were estimated. Odds ratios (ORs) were calculated with the independent variables coded in a binary form. Statistical significance was determined by Fisher' s exact test (two-tailed) and 95% confidence intervals. To derive adjusted OR (by confounders) associated with the probability of HS, multivariate unconditional logistic regression analyses were conducted. Multicollinearity in the adjusted models was tested by deriving the covariance matrix. All statistical analyses were conducted using the statistics program SPSS/PC version 12.0 (Chicago, IL, USA).

RESULTS

We enrolled 885 consecutive subjects: 319 current smokers

Table 1 Comparative characteristics between smokers and nonsmokers

Variable	Smokers (n = 319) (mean ± SD)	Non-smokers (n = 566) (mean ± SD)	P
Age (yr)	46.9 ± 11.19	46.8 ± 12.05	NS
Weight (kg)	75.5 ± 15.99	73.84 ± 14.14	NS
Gender n (%)			
Male	205 (64.26)	343 (60.60)	NS
Female	114 (35.74)	223 (38.40)	
Height (m)	1.68 ± 0.09	1.66 ± 0.09	NS
BMI (kg/m^2)	26.8 ± 4.49	26.6 ± 4.02	NS
Overweight and obesity n (%)			
BMI ⩾ 25 kg/m^2	205 (64.26)	369 (65.19)	NS
BMI ⩾ 30 kg/m^2	54 (16.93)	105 (18.55)	NS
Waist circumference (cm)	84.3 (11.2)	83.4(11.5)	NS
Glucose (mmol/L)	5.42 ± 1.53	5.42 ± 1.52	NS
Type 2 diabetes mellitus n (%)	12 (3.7)	13 (2.3)	NS
High blood pressure (> 17.33/11.33 kPa) n (%)	21 (6.6)	64 (11.3)	< 0.05
Cholesterol (mmol/L)	5.4 ± 1.14	5.33 ± 0.95	NS
C-LDL (mmol/L)	3.48 ± 0.97	3.46 ± 0.85	NS
C-HDL (mmol/L)	1.0 ± 0.26	1.06 ± 0.28	< 0.005
Triglycerides (mmol/L)	2.18 ± 1.49	1.84 ± 1.10	< 0.001
C-reactive protein (mg/L)	3.5 ± 6.03	3.4 ± 5.10	NS
Erythrocyte sedimentation rate (mm/h)	11.3 ± 9.38	13.5 ± 11.90	< 0.001
Albumin (g/L)	4.0 ± 0.28	4.0 ± 0.31	NS
Total bilirubin (mg/L)	0.9 ± 0.39	1.27 ± 8.31	NS
ALT (UI/L)	31.4 ± 20.33	33.3 ± 26.8	NS
AST (UI/L)	26.9 ± 10.25	28.9 ± 16.28	< 0.05
Metabolic syndrome n (%)	133 (41.69)	208 (36.74)	NS
ALT 2 times upper normal limit value n (%)	4 (1.25)	8 (1.41)	NS
Hepatic steatosis n (%)	87 (27.27)	168 (29.68)	NS

(205 men and 114 women) and 566 controls (343 men and 223 women). The general characteristics between groups are described in Table 1. We observed that both groups were very similar, although there were significantly more hypertensive subjects in the nonsmoker group (11.3% *vs* 6.6%, $P < 0.05$). In the current smokers group, serum levels of triglycerides were higher (2.18 ± 1.49 *vs* 1.84 ± 1.1 mmol/L, $P < 0.005$); While in contrast, they showed lower levels of HDL cholesterol (10.0 ± 206 *vs* 10.6 ± 2.8 mg/L, $P < 0.001$). Other differences between the groups were observed in erythrocyte sedimentation rate and AST values. We did not observe any differences in the prevalence of metabolic syndrome and HS. When only subjects with HS were analyzed according to smoking status, only differences in globulin values were significant (Table 2).

Univariate unconditional logistic regression analysis revealed no increased risk for HS in smoking subjects. Several models were tested, with smokers divided into subgroups depending on their smoking amount: > 10 packs/year but ⩽ 20 packs/year, and > 20 packs/year, and then compared with all other subjects and with nonsmoking subjects only (Table 3). Although there appeared to be a dose-dependent effect, this could not be confirmed. Similar analyses were made using ALT values (twice the upper normal limit value) comparing smoking and smoking intensity, which found no statistical associations (Table 4).

Table 2 Comparative characteristics in liver function tests between smokers and nonsmokers (including only subjects with hepatic steatosis)

Variable	Smokers (n = 87) (mean ± SD)	Non-smokers (n = 168) (mean ± SD)	P
Albumin (g/L)	4.07 ± 0.23	4.10 ± (0.37)	NS
Total bilirubin (mg/L)	0.94 ± 0.31	0.93 ± 0.37	NS
ALT (UI/L)	43.7 ± 24.6	49.07 ± 40.7	NS
AST (UI/L)	32.3 ± 13.0	35.2 ± 22.9	NS
Alkaline phosphatase (UI/L)	71.2 ± 18.0	74.7 ± 24.0	NS
Lactate dehydrogenase (UI/L)	154.9 ± 24	160.6 ± 30	NS
ALT 2 times upper normal limit value n (%)	2 (2.4)	8 (4.8)	NS

Table 3 Univariate model for increased risk of hepatic steatosis

Variable	OR (95% CI)
Smoking (n = 319)	0.89 (0.65-1.21)
Smoking > 10 pack/yr (n = 152)[1]	1.30 (0.88-1.92)
Smoking > 10 pack/yr (n = 137)[2]	1.16 (0.76-1.64)
Smoking > 20 pack/yr (n = 77)[1]	1.42 (0.84-2.43)
Smoking > 20 pack/yr (n = 64)[2]	1.54 (0.94-2.52)

[1] *vs* smokers less than 10 packs/yr and nonsmokers; [2] *vs* nonsmokers only.

Table 4 Univariate model for increased risk of ALT twice the upper normal limit value

Variable	OR (95% CI)
Smoking (n = 319)	0.88 (0.26-2.96)
Smoking > 10 pack/yr (n = 152)[1]	Not calculated[3]
Smoking > 10 pack/yr (n = 137)[2]	0.43 (0.05-3.39)
Smoking > 20 pack/yr (n = 77)[1]	Not calculated[3]
Smoking > 20 pack/yr (n = 64)[2]	Not calculated[3]

[1] *vs* smokers less than 10 packs/yr and non-smokers; [2] *vs* nonsmokers only; [3] Could not be calculated as there were no subjects with an ALT level twice the upper normal limit value in the specified group.

DISCUSSION

The liver is a complex organ that has numerous enzymatic pathways involved in its physiology; Many drugs can alter these delicate processes. In particular, substances derived from smoking could impair antioxidant mechanisms[11], induce cytochrome P450[18], other enzymatic pathways[19,20] and inflammatory cytokines[12]. Although the clinical importance of smoking in hepatology has been described, specifically in hepatocellular carcinoma[13], chronic infection due to hepatitis C virus[13,21] and alcoholic cirrhosis[22], less is known about NAFLD. In the present study, we found that smoking was not associated with NAFLD or elevated liver enzymes in subjects without

chronic liver disease. Recently, a prospective study by Suzuki *et al*[23] demonstrated the importance of smoking in increased levels of ALT (only in subjects who started smoking in the study); This finding supported a putative relationship between NAFLD and smoking, although in our study, the effect of chronic smoking did not appear to be significant. Indeed, such an association is implicated by data other than an epidemiological study. Currently, smoking is known to be associated with several metabolic disturbances that are considered risk factors for NAFLD. In large study cohorts, smoking increases the prevalence of diabetes mellitus, and subjects who smoke have greater IR[24], which are considered to be the hallmarks of NAFLD[2,25].

In this study, we did not find associations between smoking and smoking intensity (number of packs/year), and the prevalence of HS and ALT at twice the upper normal limit value. As the data showed both samples were very similar. It suggests that smoking does not affect directly the prevalence of NAFLD, despite our inability to evaluate insulin values and the IR index.

Another important risk factor for NAFLD is obesity, and especially the distribution of adipose tissue. The study of den Tonkelaar *et al*[9] clearly showed that smoking could influence the distribution of adipose tissue, with an increased waist/hip ratio that is considered metabolically important[26], in other nonrelated liver diseases[27-29] and NAFLD[30]. In this study, we did not observe statistical differences related to obesity, fasting serum glucose levels or prevalence of metabolic syndrome; We found minimal (but significant) differences between current smokers and nonsmokers in levels of serum triglycerides and HDLs. This indicates that the samples are very similar, and the primary variable of the study (smoking) does not influence the prevalence of NAFLD. We observed that smoking subjects had a lower prevalence of high blood pressure, where this unexpected result did not change the results in the multivariate analysis (data not shown). Similar findings are reported in samples that analyze metabolic syndrome in non-diabetic subjects[31,32]. This phenomena could be due to a long term exposure to nicotine that alters stress response resulting in a reduction in the number or affinity of receptors mediating effects of nicotine in different central nervous system structures that integrate the neuroendocrine stress response and may also lead to lower responses of other stress hormones (ACTH, prolactin, growth hormone) to a variety of stimuli[33].

However, limitations of the study should be mentioned, which could explain the lack of association between smoking and NAFLD. First, we did not evaluate IR; the information derived from this variable could partially explain our results and clarify whether smoking impairs insulin sensitivity in our population (particularly considering a potential bias because our sample includes only a selected group of subjects). The other factor that helps to explain this result is that we did not evaluate physical activity, which is another factor related to IR and possibly affected by smoking[34]; Ultrasound is non-invasive but highly unspecific in the diagnosis of NAFLD, but in large populations it does not confer ethical conflicts. Finally the low number of subjects with ALT values twice the upper normal limit value, could underpower statistical models.

In conclusion, this is the first study that has analyzed specifically the importance of smoking in NAFLD. Although some evidence suggests a role in this disease, we could not observe differences between current smokers and nonsmokers; Furthermore, no differences are found in heavy smokers (> 20 packs/year), indicating that there is no relationship between smoking and NAFLD.

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RAPID COMMUNICATION

On attitudes about colorectal cancer screening among gastrointestinal specialists and general practitioners in the Netherlands

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Abstract

AIM: To find out whether there are differences in attitudes about colorectal cancer (CRC) screening among gastrointestinal (GI) specialists and general practitioners (GPs) and which method is preferred in a national screening program

METHODS: Four hundred and twenty Dutch GI specialists in the Netherlands and 400 GPs in Amsterdam were questioned in 2004. Questions included demographics, affiliation, attitude towards screening both for the general population and themselves, methods of screening, family history and individual risk.

RESULTS: Eighty-four percent of the GI specialists returned the questionnaire in comparison to 32% of the GPs ($P < 0.001$). Among the GI specialists, 92% favoured population screening whereas 51% of GPs supported population screening ($P < 0.001$). Of the GI specialists 95% planned to be screened themselves, while 30% of GPs intended to do so ($P < 0.001$). Regarding the general population, 72% of the GI specialists preferred colonoscopy as the screening method compared to 27% of the GPs ($P < 0.001$). The method preferred for personal screening was colonoscopy in 97% of the GI specialists, while 29% of the GPs favoured colonoscopy ($P < 0.001$).

CONCLUSION: Screening for CRC is strongly supported by Dutch GI specialists and less by GPs. The major health issue is possibly misjudged by GPs. Since GPs play a crucial role in a successful national screening program, CRC awareness should be realized by increasing knowledge about the incidence and mortality, thus increasing awareness of the need for screening among GPs.

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the Western world, with over 4400 deaths per year in The Netherlands and 500 000 deaths per year worldwide[1]. Due to the aging population and population growth, the expected number of CRC patients will increase in the forthcoming years[2]. CRC is suitable for screening. Pre-malignant lesions can be identified and removed, and in case of detection in an early stage, its five year survival rate exceeds 90%[3].

The call for a national screening program in the Netherlands is increasing and implementation of the faecal occult blood test (FOBT) as a method of screening is planned within 2-3 years[4]. A meta-analysis of results from four randomised controlled trials showed that screening reduces mortality of colorectal cancer patients by 23% of those who have been actually screened[5]. However, a recent study has shown a moderate sensitivity (12%) in detecting CRC with FOBT and patient compliance of only 40%-60%[6]. Mortality reduction by means of endoscopic screening is expected to be 15%-20% higher, but evidence from prospective randomized trials is lacking[7].

The Netherlands rank the lowest public awareness regarding CRC among countries in Europe[8]. In some countries feasibility and implementation studies of nationwide screening programs, together with capacity inventories, are ongoing, while Dutch reports are still awaited[9-11].

A high participation level of the general population is essential for a national screening program to succeed. The key to achieving adequate compliance is informing the community through an educational campaign about the nature and extent of the disease, as well as education re-

Table 1 Population and personal screening

	GI specialists (%)	GPs (%)	Fisher exact test
In favour of population screening	92	51	$P < 0.001$
In favour of personal screening	95	30	$P < 0.001$

Table 2 Favoured method of population screening

	GI specialists (%)	GPs (%)	Fisher exact test
FOBT	0	26	$P < 0.001$
Sigmoidoscopy	0	18	$P < 0.001$
Combination FOBT + sigmoidoscopy	12	0	$P < 0.001$
Colonoscopy	72	27	$P < 0.001$
Fecal DNA test/ CT-colonoscopy	16	19	NS ($P = 0.49$)
Indifferent/Unknown	0	10	$P < 0.001$

Table 3 Age at initiation of screening

	GI specialists (%)	GPs (%)	Fisher exact test
50 yr	37	29	NS ($P = 0.39$)
55 yr	42	18	$P = 0.005$
60 yr	21	13	NS ($P = 0.29$)
Unknown	0	40	$P < 0.001$

Table 4 Familial predisposition to CRC and personal judgment of increased risk of developing CRC

	GI specialist (%)	GPs (%)	Fisher exact test
Familial predisposition for CRC[1]	4	25	$P < 0.001$
Personal judgment of increased risk for CRC	2	8	$P = 0.006$

[1] Defined as one or more first degree relatives diagnosed with CRC.

garding the method of screening to be used. Involvement of the general practitioners (GPs) is crucial in increasing the yield of a CRC screening program with FOBT[12-14].

There is ongoing debate among physicians and politicians on the necessity of CRC screening and which screening modality is to be used. This study was to inquire into the attitudes regarding screening among Dutch gastroenterologists, gastrointestinal (GI) surgeons and GPs.

MATERIALS AND METHODS

During a biannual meeting in 2004 in the Netherlands, addressing all general topics in gastroenterology, all the attending Dutch gastroenterologists and gastrointestinal surgeons were questioned ($n = 420$). The same 17-item questionnaire was sent to all GPs in Amsterdam ($n = 400$) in fall, 2004. In an attempt to achieve a comparable sample size between GI specialists and GPs, only GPs from Amsterdam were asked to return the questionnaire. Questions included demographics, affiliation, attitude towards screening both for the general population and themselves, methods of screening, family history and individual risk.

Statistical analysis

SPSS for Windows version 11.0 was used for the descriptive statistical analysis. The Fisher exact test was used for the comparison of proportions. $P < 0.05$ was considered statistically significant.

RESULTS

Responses were obtained from 354 of the 420 GI specialists (84%) who were questioned. Among the GI specialists 82% were gastroenterologists and 18% gastrointestinal surgeons. Eleven percent of the GI specialists worked in an academic setting and 89% in a community hospital. The mean age of GI specialists was 48 years (range 28-71 years). The response rate among the 400 potentially eligible GPs was 32% (126). All GPs worked in Amsterdam. The mean age of the GPs was 49 years (range 32-69 years).

A highly significant difference in appreciation of population and personal screening was found between GI specialists and GPs ($P < 0.001$, Table 1). Moreover, significant differences were found in the preferred screening method. Colonoscopy was considered the primary population screening tool by the majority of GI specialists, while FOBT, sigmoidoscopy and colonoscopy were almost equally supported by GPs (Table 2). Personal screening with colonoscopy was favoured by 97% and 27% of GI specialists and GPs, respectively ($P < 0.001$). Forty-two percent of GI specialists considered 55 years as the proper age to start personal screening, whereas the age of 50 years was chosen by GPs (Table 3). Four percent of GI specialists and 25% of GPs reported a familial predisposition to CRC. Nevertheless, within the latter two groups only 50% and 33% regarded themselves at a higher risk of developing CRC (Table 4). Finally, a subgroup analysis was performed on the GPs with familial predisposition to CRC, regarding their opinion on population screening. In this group 61% preferred population screening, compared to 51% in the total group of GPs ($P = 0.32$).

DISCUSSION

Population screening for CRC is strongly supported by Dutch GI specialists. In their opinion benefits definitely outweigh the drawbacks and their discussion focuses on how to implement a national CRC screening program and which method should be used. Unfortunately, the above-mentioned results suggest that GPs are more reluctant to speak out in favour of a CRC population-based screening program than GI specialists. Only 51% of responding GPs are in favour of population screening. A challenging task is reserved for the GI specialists to convince GPs of the need for screening. It has been shown that by increasing

knowledge about the incidence and mortality of CRC, as well as the possibility for early detection, the attitudes of GPs will change[15].

There is a remarkable difference in returned questionnaires between GI specialists and GPs. It can be hypothesized that completing a short questionnaire on a meeting is less of a burden than during daily routines, where other priorities may prevail. However, the response rates differ significantly, suggesting that GPs are ignorant of the CRC screening issue or are in a low state of awareness of the problem' s magnitude. These findings correspond to the previous reports stating a lack of interest and knowledge in the definition of the high risk population among GPs[16,17].

Another argument may be that only half of all GPs favour population screening and only 30% intend to be screened themselves. In the present study, no significant difference was observed in preference of population screening (61% *vs* 51%; $P = 0.32$) even between a subgroup of GPs with a familial predisposition to CRC which results in an increased risk of developing CRC and the total group of GPs. In contrast, 92% of all the GI specialists supported such a nation-wide CRC screening program and 95% planned to undergo personal screening. In this context, it can be put forward that knowledge of the natural course of CRC, its pre-malignant precursor lesions and therapeutic options in case of early detection, might explain the large differences in opinion. Since GPs play a crucial role in achieving a successful national screening program, the latter suggestion should be a concern to GI specialists and central government. On the other hand, GPs are subjected to a continuous barrage about the different types of screening (lung, breast, prostate, CRC, cervix) and they might have a wider view on priorities and cost-efficiency in the health sector.

Regarding the screening modalities for both personal and population screening, a clear preference to colonoscopy was observed among GI specialists.

Colonoscopy is the method of screening preferred by GI specialists. In the present study, 97% of the GI specialists preferred this method for their personal screening, and 72% for population screening. The higher preference to alternative methods for population screening presumably reflects concerns regarding the capacity and logistics (Table 2).

Among GPs, population screening with FOBT or colonoscopy was equally supported (respectively 26% and 27%). A rather surprising finding is that none of the GI specialists preferred FOBT as a screening method, since there is evidence in terms of cost-effectiveness and mortality reduction[18-20]. Nevertheless, the fact that GI specialists agree with and encourage the implementation of FOBT for nationwide screening can be explained by the fact that FOBT is the only screening method accepted by the central government at this moment. Furthermore, there is a convincing mortality reduction using FOBT as a method of screening and in this perspective screening with FOBT is better in any case than no screening[5,18]. In addition, an ongoing screening program allows future alternatives to be implemented more easily, when they are proven superior to FOBT.

Finally, a large proportion of GI specialists plan to start personal screening at the age of 50-55 years, even though only 4% are found at a high risk of developing CRC. In 40% of responding GPs in favour of personal screening, the age at which screening should be initiated is unknown (Tables 3 and 4). This emphasises the importance of education about this disease since one out of 20 people will develop CRC during a lifetime, with advancing years of age as the foremost risk factor for CRC development. The highest mortality rate for CRC appears to be around the age of 60 years, the time interval in which a precursor lesion develops into an invasive cancer is 10-15 years[21].

In conclusion, the findings of our study are relevant to GPs in Amsterdam. However it is unknown whether GPs in other parts of the Netherlands have similar attitudes. Nonetheless, this inquiry clearly indicates the urgent need for GI specialists and GPs to solve the ongoing debate on CRC screening. Education of all parties involved should lead to an increased knowledge about the magnitude of the CRC problem. An unambiguous policy stressing the crucial role of GPs in a CRC screening program, may improve patient compliance, thereby reducing the mortality of CRC. More studies are mandatory to draw firm conclusions.

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RAPID COMMUNICATION

Association of *H pylori cagA and vacA* genotypes and IL-8 gene polymorphisms with clinical outcome of infection in Iranian patients with gastrointestinal diseases

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Abstract

AIM: To find out if a functional promoter polymorphism in the IL-8 gene along with *cagA* status and polymorphisms in *vacA* gene influence the type of diseases in Iranian patients infected by *H pylori*.

METHODS: IL-8 -251 A/T polymorphism was genotyped by oligonucleotide allele specific PCR (ASO-PCR) in a sample of 233 patients with *H pylori* infection undergoing upper gastrointestinal endoscopy. The presence of *cagA* gene and polymorphisms in *vacA* gene was also determined by PCR. Association of these genetic polymorphisms with the development of gastritis, peptic ulcers as well as gastric cancer was tested.

RESULTS: When the patients with different clinical manifestations were compared according to the presence of *cagA* gene or various *vacA* genotypes, only the *vacA* genotypes were significantly different among gastritis, peptic ulcer and gastric cancer patients (χ^2 = 17.8; P = 0.001). Furthermore, there was a significant difference in the frequency of IL-8 -251 A/T genotypes between patients with gastric cancer and benign diseases (χ^2 = 10.47; P = 0.005).

CONCLUSION: The IL-8 -251 A/T polymorphism and the polymorphisms in *H pylori vacA* gene are involved in limiting the infection outcome to gastritis and peptic ulcer or in favoring cancer onset in Iranian patients.

Key words: Interleukin 8; *H pylori*; Gastric cancer; Peptic ulcer; Polymorphism

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INTRODUCTION

Infection with *H pylori* has plausible associations with a variety of clinical outcomes, including chronic gastritis, peptic ulcer and gastric cancer[1-4]. Variation in the clinical outcome of *H pylori* induced pathology is multifactorial, involving a complex interplay between the host immune responses, pathogen virulence factors, and niche characteristics. Many putative virulence factors have been identified in *H pylori* that contribute to its pathogenesis. The 128-kDa cytotoxin-associated gene encoded antigen A (*cagA*)[5] and vacuolating cytotoxin antigen gene (*vacA*)[6] are known as the most important ones. *cagA* gene was identified as a strain-specific *H pylori* gene and has been recognized as a marker for strains that confer increased risk for peptic ulcer disease[7-8] and gastric cancer[9]. The *cagA* gene is present downstream of a 40-kb cluster of virulence genes known as the *cag* pathogenicity island (*cag*-PAI). These genes encode a type Ⅳ secretion system that forms a syringe-like structure to translocate the immunodominant *cagA* protein into the gastric epithelial cells. *cag*-PAI has also been implicated in the induction of IL-8 in cultured gastric cells[10]. This property contributes to the proinflammatory power of the strains and thus

to their virulence capability. The difference between *H pylori* strains in virulence capability is also dependent on the expression of *vacA* (87 kDa), which is toxic to epithelial cells *in vitro*[11-13]. Moreover, mice which were administered *vacA* orally developed gastric ulcers[14]. Unlike the *cagA*, *vacA* gene is conserved among all *H pylori* strains, although significant polymorphism exists in its gene[15]. *vacA* alleles possess one of two types of signal regions, s1 or s2, and one of two mid-regions, m1 or m2, occurring in all possible combinations. The *vacA* signal region encodes the signal peptide and the N-terminus of the processed *vacA* toxin: type s1 *vacA* is fully active, but type 2 has a short N-terminus extension that blocks vacuole formation[16]. *vacA* s2 strains are rarely isolated from patients with peptic ulcers or gastric adenocarcinoma[15]. The *vacA* mid-region encodes part of the toxin cell binding domain. Vacuolating activity is higher in s1/m1 genotypes than in s1/m2 genotypes, and is absent in s2/m2 genotypes[16]. Consequently, *vacA* s1/m1 genotypes are more frequently associated with peptic ulceration and gastric carcinoma[17,18]. The genetic heterogeneity in immune responses among individuals is another important factor which determines the clinical outcome of *H pylori* infection. Support for this consideration is provided by the low frequency of gastric cancer in some developing countries in spite of the paradoxically high prevalence of *H pylori* infection in those countries[19,20]. Up to now, there are several reports indicating the association of IL-1β, tumor necrosis factor α (TNF-α), and IL-10 gene polymorphisms with an increased risk of developing gastric atrophy, hypochlorhydria, and non-cardia gastric cancer[21-23]. Due to the roles which are played by IL-8 in the pathogenesis of *H pylori* infection, the IL-8 gene is one of the most important candidate host genes in determination of the outcome of *H pylori* infection. This cytokine is produced by gastric epithelial cells as an early response to *H pylori* virulence factors, such as *cagA*[10]. IL-8 is also a major host mediator involved in neutrophil and phagocyte chemotaxis and activation[24,25], thereby causing mucosal damage by releasing reactive oxygen radicals[25]. It is therefore tempting to speculate that mucosal IL-8 production due to *H pylori* infection may be an important factor in the immunopathogenesis of peptic ulcer diseases and may also be relevant in gastric carcinogenesis[26]. Interestingly, previous studies have suggested that the production of IL-8 is genetically determined and neutrophils from individuals who are homozygous for the AA genotype at the -251 position demonstrated a trend toward higher levels of IL-8 production in response to lipopolysaccharides than those without the allele[27]. More recently, Ohyauchi *et al* reported that IL-8 -251 A/T polymorphism influences the susceptibility of *H pylori* related gastric diseases in the Japanese population[28]. Furthermore, in an *H pylori* infected Chinese population the risk of gastric cancer was also significantly elevated in patients with the IL-8-251 AA genotype[29]. Considering the above information, the aim of this study was to look for an association between IL-8 -251 A/T polymorphism, *vacA* genotypes, the presence of *cagA* gene and clinical outcome of *H pylori* infection in Iranian patients.

MATERIALS AND METHODS

Patients and Bacterial strains

In the present study 298 patients were classified at the time of endoscopy into those having gastritis (n = 199), gastric ulcer (n = 12), duodenal ulcer or duodenitis (n = 67) and non-cardia gastric carcinoma (n = 20). This classification was also confirmed by histological examinations. These patients have been referred for upper gastrointestinal endoscopy to the Gastroenterology Section of the University Hospitals (Namazi and Shahid Faghihi) of Shiraz University of Medical Sciences between 2002 and 2005. Patients who had received non-steroidal anti-inflammatory drugs were excluded. *H pylori* strains were successfully isolated from the gastric biopsies of 286 patients (150 males, 136 females; median age 45.3 ± 16.6 years). The present study was approved by the local ethics committee.

Bacterial culture and histological examination

Biopsy specimens were taken from the antrum and corpus of the stomach. These specimens were used for the rapid urease test, bacterial culture, and histological assessment. After 5 d of culture on selective agar plates, the organisms were identified as *H pylori* by Gram staining, colony morphology, and positive oxidase, catalase and urease reactions.

Preparation of patients and H pylori genomic DNA

After 3-5 d of culture, *H pylori* colonies were pooled from the plates and washed using phosphate-buffered saline. *H pylori* genomic DNA was prepared after bacterial cell lysis using SDS and proteinase K solution and phenol-chloroform extraction. Patients genomic DNA was extracted from EDTA anticoagulated peripheral blood leucocytes using a salting out method. The DNA samples were maintained at -70℃ until use in polymerase chain reaction (PCR).

Analysis of IL-8 and bacterial vacA and cagA genotypes

All primer sets used were selected from the published literature and are shown in Table 1. An allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) was used to detect the polymorphism at position -251 of the IL-8 gene[30]. As an internal control, the β-globin specific primers were included in the ASO-PCR (Table 1). For IL-8 genotyping, 10 μL of PCR reaction mixture consisting of 250 ng of genomic DNA, 200 μmol/L dNTPs, 2.25 mmol/L $MgCl_2$, 1 × Taq DNA polymerase buffer, 2 units of Taq DNA polymerase (Boehringer Manheim, Germany), 10 pmol of each test primer and 5 pmol of internal control primers were employed. Then, a touch-down procedure was followed that consisted of 25 s at 95℃, annealing for 45 s at temperatures decreasing from 68℃ (four cycles) to 61℃ (20 cycles), and an extension step at 72℃ for 40 s. The annealing temperature for the remaining 5 cycles was 58℃ for 40 s. For *cagA* and *vacA* genes the PCR was performed using a thermal cycler (Master Cycler; Eppenderof, Germany) under the following conditions: an initial denaturation for 5 min at 94℃, 35 cycles of 60 s at 94℃, 60 s at 57℃ and 60 s at

Table 1 Primer sequences used for detection of *cagA* gene status and IL-8 or *vacA* gene polymorphisms

Locus	Primers	Size (bp)
IL-8 -251	*Common primer, 5′-tgc ccc ttc act ctg tta ac-3′*	336
	A allele, 5′-cca caa ttt ggt gaa tta tca at-3′	
	T allele, 5′-cca caa ttt ggt gaa tta tca aa-3′	
β-globin	*5′- aca caa ctg tgt tca cta gc -3′*	100
	5′- caa ctt cat cca cgt tca cc -3′	
*cag*A	5′-aat aca cca acg cct cca ag-3′	400
	5′-ttg ttg ccg ctt ttg ctc tc-3′	
*vac*A		
S1 and s2	5′-ctg ctt gaa tgc gcc aaa c-3′	s1 = 259
	5′-atg gaa ata caa caa aca cac-3′	s2 = 286
m1 and m2	5′-gcg tct aaa taa ttc caa gg-3′	m1 = 570
	5′-caa tct gtc caa tca agc gag-3′	m2 = 645
glmM	5′-aag ctt tta ggg gtg tta ggg gtt t-3′	294
	5′-aag ctt act ttc taa cac taa cgc-3′	

72℃, with a final extension step at 72℃ for 5 min. The PCR system for *cagA* contained 10 × PCR buffer, 2.5 μL; $MgCl_2$, 1.2 mmol/L; dNTP, 200 μmol/L; *cagA* specific primers, 10 pmol; Taq DNA polymerase, 1.0 U; and the DNA template, 50 ng. To confirm the identification of the bacteria as *H pylori*, 10 pmol of *glmM* specific primers were included in each PCR reaction for *cagA* gene (*glmM* is a conserved gene formerly known as *ureC*). For detection of *vacA* polymorphisms a multiplex PCR was performed using the specific primers for amplification of s and m genes (Table 1). The PCR system for *vacA* genotyping was similar to *cagA* genotyping. PCR products were examined by 2% agarose gel electrophoresis and photographed using an ultraviolet reflection analyzer.

Statistical analysis

Fisher's exact test and the χ^2 test were used to analyze the data from different disease groups. All tests were performed two tailed with a confidence interval (CI) of 95%. A *P*-value of less than 0.05 was accepted as statistically significant. The Statistical Package for the Social Sciences (SPSS) version 11.5 was used for statistical analysis.

RESULTS

Prevalence of the cagA and vacA gene in different disease groups

Table 2 shows the distribution of the 286 strains according to their *cagA* and *vacA* types in different patient groups. To detect the *cagA* subtype of *H pylori*, *cagA* specific PCR was performed on extracted bacterial DNA from patients with gastric diseases. Amplified *cagA* DNA fragments were detected in 219/286 (76.6%) of our *H pylori*. Sixty seven patients (23.4%) were *cagA*-, although they were *glmM*+ or *vacA*+. When the patients with different clinical manifestations were compared according to the presence of the *cagA* gene (Table 2), insignificant differences in *cagA* status were found among patients with peptic ulcer, gastritis and gastric cancer (χ^2 = 1.9; *P* = 0.38). Also, when the frequency of *cagA*+ and *cagA*- subtypes were compared among patients with gastritis and gastric ulcer

Table 2 IL-8 -251 A/T polymorphism, *cagA* status and *vacA* gene polymorphism in patients with gastric diseases

Locus	Genotype	Patients Gastritis *n* (%)	Peptic Ulcer *n* (%)	Gastric Cancer *n* (%)	*P* value
IL-8 -251	AA	22 (14.4)	14 (23.0)	9.0 (47.4)	0.013
	AT	74 (48.4)	28 (45.9)	6.0 (31.6)	
	TT	57 (37.3)	19 (31.1)	4.0 (21.1)	
*cag*A	+	148 (74.4)	54 (80.6)	17 (85.0)	0.39
	-	51 (25.6)	13 (19.4)	3.0 (15.0)	
*vac*A	s1m1	48 (26.5)	21 (32.3)	12.0 (66.7)	0.001
	s1m2	74 (40.9)	33 (50.8)	3.0 (16.7)	
	s2m2	59 (32.6)	11 (16.9)	3.0 (16.7)	

(χ^2 = 1; *P* = 0.3), gastritis and peptic ulcer (χ^2 = 0.75; *P* = 0.38), gastritis and cancer (χ^2 = 0.61; *P* = 0.42) or between peptic ulcer and gastric cancer (χ^2 = 0.01; *P* = 0.75), the difference always remained insignificant. In the case of *vacA* gene, three of the four possible combinations of signal sequence and middle-region types were identified. The s1/m1 type was found in 81/264 (30.7%) of the isolates, the s1/m2 type in 110/264 (41.7%) of the isolates, and the s2/m2 type in 73/264 (27.7%) of the isolates. The distribution of *vacA* genotypes (s1/m1, s1/m2 or s2/m2) were significantly different among peptic ulcer, gastritis and gastric cancer patients (χ^2 = 17.8; *P* = 0.001). Interestingly, the difference in distribution of three different *vac*A genotypes between patients with gastric cancer and gastritis (χ^2 = 12.57; *P* = 0.0018) was more significant than the difference in distribution of *vac*A genotypes between patients with gastric cancer and peptic ulcer (χ^2 = 7.97; *P* = 0.018). In fact, the frequency of s1m1 genotype was notably higher in gastric cancer patients (66.7%) compared to those with gastritis or peptic ulcer (26.5% and 32.3%, respectively). Furthermore, similar to previous reports[15], there is a strong statistical linkage between the s1 genotype of *vacA* and the presence of the *cag* island (χ^2 = 27.95; *P* = 0.0000001; OR = 4.99, 95% CI = 2.61-9.58). Similarly, the s2 genotype is associated with the lack of the *cag* island. In fact, strains that are *cag*+ are more likely to possess the *vac*A s1 allele than *cag*- strains.

Prevalence of the IL-8 -251 A/T polymorphism in different disease groups

The allelic frequencies of IL-8 -251 A/T polymorphism and genotype distributions are given in table 2. The IL-8 -251 A/T polymorphism showed no evidence of deviation from the Hardy-Weinberg equilibrium, with a non-significant χ^2 value (χ^2 = 0.05, *P* = 0.4). Interestingly, there was a significant difference in the frequency of IL-8 -251 A/T genotypes between patients with gastric cancer and those with benign diseases (χ^2 = 10.47; *P* = 0.005). Moreover, when the patients were categorized to high producer (AA) and intermediate + low producer (AT + TT) genotypes, a more meaningful difference in the frequency of IL-8 -251 A/T genotypes between patients with gastric cancer and benign diseases was noticeable (OR = 4.45, 95% CI = 1.53-12.94; χ^2 = 8.58, *P* = 0.003). In fact, the prevalence of AA genotype in gastric cancer

patients was 47.4% compared to 16.8% in benign diseases. While comparing different patient groups, no significant differences were demonstrated in frequencies of IL-8 -251 A/T genotypes between patients with peptic ulcer and gastritis ($\chi^2 = 2.4$; $P = 0.25$) or gastric cancer ($\chi^2 = 4.22$; $P = 0.12$). However, IL-8 -251 A/T polymorphism showed a significant difference between patients with gastric cancer and gastritis ($\chi^2 = 12.5$; $P = 0.001$).

DISCUSSION

After exposure to *H pylori*, the clinical manifestations are variable and depend on host and pathogen factors. There is no information on the prevalence and role of *H pylori* genotypes and/or the role of IL-8 -251 A/T genotypes in the disease outcome of Iranian patients. Therefore, in the present study, we determined the presence of the *cagA* gene (as a marker of *cag* pathogenicity island) and the genotypes of *vacA* gene in the infecting strains along with the distribution of host IL-8 genotypes in relation to the occurrence of different clinical manifestations in Iranian patients with *H pylori* infection. It has been shown that exposure of gastric epithelial cells to *cag*+ *H pylori* strains can activate the proto-oncogenes c-*fos* and c-*jun*, a crucial step in the development of *H pylori*-related neoplasia[31]. The presence of *cagA* has been statistically associated with duodenal ulceration, gastric mucosal atrophy, and gastric cancer[7-9], although some studies deny this association[32-34]. Audibert and her colleagues reported that *cagA* status is not sufficient to predict the IL-8 induction ability of *H pylori* and is not correlated with the presence or absence of ulcer[35]. In a series of patients from Taiwan, the presence of *cagA* gene in the PAI also showed no relationship to the type of disease and/or the histological features of the patients[36]. The results of the present study also shows that the prevalence of *cagA* is not significantly different among different disease groups ($P > 0.05$), which is in accordance with the results of other Asian countries. Therefore, the *cag*-PAI may not be the principal virulence factor, as suggested by the absence or sporadic distribution of the *cag*-PAI genes among strains from varied clinical outcomes. However, considering the high prevalence of *cag*+ strains in Iranian patients (76.6%), the relationship of *cag* status with disease type is more difficult to establish in our population. Therefore, larger sample sizes are recommended for such studies.

Furthermore, the lack of association may be due to the fact that the development of cancer or ulcer disease is a complex process that also involves factors other than the *cag*-PAI, such as *vacA*. Certain *vac*A genotypes causing a high vacuolating activity are correlated with increased disease severity in humans[15]. Several studies have also shown that the presence of *vacA* is associated with peptic ulcer diseases[14,17-18]. The *vacA* gene displays a considerable polymorphism, especially in the signal region (genotypes s1 and s2) and in a mid region (genotypes m1 and m2). Vacuolating activity is higher in s1/m1 genotypes than in s1/m2 genotypes, and is absent in s2/m2 genotypes[15]. Consequently, *vacA* s1/m1 strains cause more direct epithelial damage and are more frequently associated with peptic ulceration and gastric carcinoma[9,17,18]. The results of the present study also showy a significant difference in *vac*A genotype distribution between gastric cancer and gastritis ($P = 0.0018$) or peptic ulcer ($P = 0.018$) patients. The marked increase of s1m1 genotype in gastric cancer patients (66.7%) compared to those in patients with gastritis (26.5%) or peptic ulcer (32.3%) confirms the pathogenic role of this virulence determinant in Iranian patients. However, different disease outcomes were encountered in subjects infected with *H pylori* strains sharing the same virulent *vac*A genotype, s1m1. The different outcomes of *H pylori* infection may depend not only on other bacterial factors but also on the different genetic background of the host. Concerning host genetic factors, Thye *et al* performed a genome wide screen analysis to identify the genetic factor(s), which define susceptibility to *H pylori* infection[37] and suggested the presence of a possible linkage with chromosomes[4]. Considering the location of the human IL-8 gene on chromosome 4 (4q13-q21), the results of their study may support the hypothesis that the IL-8 gene polymorphism is probably associated with *H pylori* induced gastrointestinal diseases. Interestingly, the inheritance of the IL-8 -251A allele was associated with progression of gastric atrophy in patients with *H pylori* infection and increased the risk of gastric cancer in Japanese and Chinese people[28,29,38]. Our results also indicate that gastric cancer is significantly associated with the functional polymorphism in the promoter region of the IL-8 gene. Specifically, individuals genetically predisposed to produce more IL-8 are at a higher risk of developing gastric cancer. The finding that there was an increased risk of gastric cancer in high IL-8 producers was in agreement with the concept that IL-8 may influence the risk of developing gastric cancer by altering the quality and vigor of inflammatory responses produced by the host after exposure to *H pylori*. In addition, IL-8 stimulated neutrophils to synthesize active radicals such as nitric oxide[25]. These radicals by their mutagenic potential[39] could cause mutations in gastric epithelial cells. In addition, IL-8 by inducing angiogenesis would be one of the important factors in gastric carcinogenesis. In support of this hypothesis, the expression of IL-8 has been associated with increased vascularization and poor prognosis in gastric carcinoma[40,41]. Thus, inheritance of the high producer allele of IL-8 (carriers of -251 A allele) may induce chronic gastritis, which may then be followed by the development of gastric cancer.

In conclusion, similar to studies performed in China and Japan, the association between *cag*A positivity and virulence of *H pylori* strains was equally frequent among Iranian patients with different disease types. Moreover, the present study provides further evidence that in addition to genetic polymorphism of the *vac*A gene in the pathogen, genetically determined differences in IL-8 production via promoter polymorphisms could contribute to individual susceptibility to gastric cancer development after *H pylori* infection in Iranian patients.

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RAPID COMMUNICATION

Preliminary report of hepatitis B virus genotype prevalence in Iran

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Abstract

AIM: To determine the prevalence of hepatitis B virus (HBV) genotypes in Iranian hepatitis B surface antigen (HBsAg) carriers, chronic hepatitis B and cirrhotic patients.

METHODS: A total of 109 HBsAg-positive patients were included in this study. HBV genotypes were determined by using INNO-LiPA methodology which is based on the reverse hybridization principle.

RESULTS: The distribution of patients with different stages of liver disease was as follows: 95 (86.4%) chronic hepatitis, 11 (10%) liver cirrhosis, and 3 (2.7%) inactive carrier. Of the chronic hepatitis and liver cirrhosis patients, 26.4% were HBeAg-positive while 70% were HBeAg-negative. Genotype D was the only detected type found in all patients.

CONCLUSION: Classifying HBV into genotypes has to be cost-effective and clinically relevant. Our study indicates that HBV genotype D prevails in the Mediterranean area, Near and Middle East, and South Asia. Continued efforts for understanding HBV genotype through international co-operation will reveal further virological differences of the genotypes and their clinical relevance.

Key words: Hepatitis B virus; Genotype; Chronic Hepatitis B; Cirrhosis

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INTRODUCTION

Human hepatitis B virus (HBV), which is the prototype member of the family Hepadnaviridae, is a circular, partially double-stranded DNA virus of approximately 3200 nt[1]. This highly compact genome contains the four major open reading frames encoding the envelope (preS1, preS2 and surface antigen HBsAg), polymerase (HBPol) and X (HBX) proteins, respectively[2]. HBV is an etiologic agent of acute and chronic liver disease, including fatal fulminant hepatitis, liver cirrhosis and hepatocellular carcinoma[3-6]. Over 2 billion people worldwide have been exposed to HBV and 350 million are chronic carriers of HBV[7-9].

In 1988, HBV was classified into four genotypes by a sequence divergence in the entire genome exceeding 8%, and designated by capital letters of the alphabet from A to D[10,11]. In 1994, Norder *et al*[12] found an additional two HBV genotypes by the same criteria, and named them E and F, respectively. Genotype G was reported recently in 2000[2] and genotype H, which is phylogenetically closely related to genotype F, was proposed in 2002[11]. HBV genotypes have distinct geographical distribution[2,7,13,14].

In general, genotype A is pandemic, but most prevalent in North West Europe, North America, Central Africa[2,13] and India[7]. Genotypes B and C are prevalent in Asia[7,8,13,15], especially in populations of Eastern Asia and the far East origin[3,16]. Genotype D is also more or less pandemic, but is predominant in the Mediterranean area and the Middle East[2,3,16]. Genotype E is restricted to Africa and genotype F is found in Central and South America[7,8,13,16]. Genotype G has been recently identified in France and North America[7,8]. It has been reported that there are remarkable differences in the clinical and virologic characteristics between the patients with different genotypes[17,18,19]. According to Iranian studies[20], over 35% of Iranians have been exposed to HBV, approximately 2% are chronic carriers. Compared to the United States where HBV infection is responsible for 25% of chronic hepatitis, HBV accounts for up to 70%-80% of chronic hepatitis cases in Iran, indicating that HBV alone is the leading

cause of chronic liver disease in Iran[21]. Until now, to our knowledge, no data regarding HBV genotype is available, and also the genotypes distribution of HBV and genotype-related differences with the liver disease are still unclear in Iran. In this study, we therefore determined the prevalence of HBV genotypes in Iranian hepatitis B surface antigen (HBsAg) carriers, chronic hepatitis B and cirrhotic patients.

MATERIALS AND METHODS

This cross-sectional study was performed in Tehran Hepatitis Center in 2004. A total of 109 patients with hepatitis B surface antigen (HBsAg) positive for at least 6 mo were enrolled in this study. Of the 109 patients, 95 were classified as chronic hepatitis, defined as HBsAg positivite with or without the presence of HBeAg, high level of HBV-DNA (> 100 000 copies/mL) detected by Amplicor HBV monitor, persistent or intermittent elevation in ALT levels and compatible liver biopsy. Three were inactive carriers characterized by persistent HBV infection of liver without significant, ongoing necro-inflammatory disease. Eleven had liver cirrhosis characterized by clinical evidence (splenomegaly, ascitis) and paraclinical results, including low platelet count, prolongation of prothrombin time and esophageal varices on upper gastrointestinal endoscopy. Liver cirrhosis was confirmed by liver biopsy. Patients were excluded if they were co-infected with hepatitis C virus (HCV), hepatitis D virus (HDV) or human immunodeficiency virus (HIV).

The following parameters were recorded for each patient from patient's document in Tehran Hepatitis Center: sex, age, stage of liver disease, alanine amino-transferase (ALT) level, aspartate transaminase (AST) and presence of hepatitis B virus E antigen and anti-hepatitis B virus E antibodies.

HBV genotypes were determined by using INNO-LiPA methodology (LiPA, INNO-LiPA HBV genotyping assay, Innogenetics N.V., Ghent, Belgium). The INNO-LiPA HBV genotyping assay is a line probe assay designated to identify hepatitis B virus genotypes A to G by detection of type-specific sequences in the HBV-pol gene domain B to C. This method is based on the reverse hybridization principle. Biotinylated DNA material generated from the HBsAg open reading frame was hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane-based strips. After hybridization, unhybridized DNA was washed from the strip, alkaline phosphatase-labeled streptavidin was added and bounded to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen resulted in a purple/brown precipitate. Amplification of appropriate the HBV genomic region was performed using the INNO-LiPA HBV DR amplification kit. The INNO-LiPA HBV genotyping strip contains 1 red marker line, 2 control lines, and 14 parallel probe lines. The conjugate control line is a control for the color development reaction and the amplification control line contains universal HBV probes to check for the presence of amplified the HBV genomic material.

Statistical analysis

Data were analyzed with SPSS 11.5 software (SPSS Inc. Chicago, Illions, USA) using Student's t test, χ^2 test and Fisher's exact test.

RESULTS

A total of 109 patients with a mean age of 37.17 ± 11.75 years, including 13% females and 87% males, were enrolled in this HBV genotype study. The distribution of patients in different stages of liver disease was as follows: 95 (86.4%) chronic hepatitis, 11 (10%) liver cirrhosis, and 3 (2.7%) inactive carrier. Of the chronic hepatitis and liver cirrhosis patients, 26.4% were HBeAg-positive and 70% were HBeAg-negative. The mean serum ALT, AST, and ALP levels were 126.08 IU/L (88.46-163.71), 86.46 IU/L (49.54-123.39), 173.34 IU/L (152.74-193.94), respectively.

Genotype D was the only detected type found in all patients. Mean age of patients was significantly higher in the anti-HBe-positive group as compared with the HBe-Ag-positive group (P = 0.000). Also, the number of the patients in the anti-HBe-positive group was significantly higher than the HBe-Ag-positive group (P = 0.019; Fisher' s exact test). Moreover, significant difference was found between the mean age of patients with different stages of liver disease. None of the patients in the HBe-Ag-positive group had a normal ALT level. Most of the patients in the chronic hepatitis stage had an abnormal ALT level in comparison with the liver cirrhosis stage (P = 0.024; Fisher's exact test).

DISCUSSION

Classifying HBV into genotypes has to be cost-effective and clinically relevant. It is imperative to collect more information on HBV genotypes from all over the world to reach a decision on their clinical utility[11].

Data on the relation among the HBV genotypes, their pathogenicity in chronic liver disease including hepatocellular carcinoma and their effect on therapy are awaited with great interest, especially in Asia which is an endemic region of blood-borne hepatitis viruses[15].

Presently, based on an intergroup divergence of 8% or more in the complete nucleotide sequence, HBV can be classified into eight genotypes A-H, and different HBV genotypes are dominant in various parts of the world[14,16]. The most important finding of our study was that the only HBV genotype D was detected in all patients. The pattern of distribution of genotypes seemed to be simpler and was predominantly centralized into genotype D in all forms of the chronic HBV infection. The results of this study concur with previous studies, indicating that HBV genotype D prevails in the Mediterranean area, Near and Middle East, and South Asia[3,4,16]. For example, the result of a similar study performed in Turkey showed all 44 patients studied had genotype D[16]. Another study in Yemen demonstrated that genotype D was the dominant genotype in a settled population, while genotype A was

found only in communities with continuing African links[22]. In addition, one study in Egypt revealed genotype D was the most prevalent HBV genotype[23]. On the contrary, genotypes A, B and C were found to be predominant in Pakistan[24]. According to recent studies, genotype D in Asia is associated with more severe disease and may predict occurrence of hepatocellular carcinoma in younger patients[9].

After all, only less than 1 000 of the 350 million persistent HBV infections have yet been genotyped. Continued efforts for understanding HBV genotypes through international co-operation will reveal further virological differences of the genotypes and their clinical relevance.

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RAPID COMMUNICATION

Effect of Fructus Psoraleae on motility of gallbladder isolated smooth muscle strips from guinea pigs

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Abstract

AIM: To observe the effect of Fructus Psoraleae on motility of isolated gallbladder muscle strips of guinea pigs and its mechanism.

METHODS: Guinea pigs were hit to lose consciousness and the whole gallbladder was removed quickly. Two or three smooth muscle strips (8 mm × 3 mm) were cut along a longitudinal direction. The mucosa was gently removed. Every longitudinal muscle strip was suspended in a tissue chamber which was continuously perfused with 5 mL Krebs solution (37℃), pH 7.4, and aerated with 950 mL/L O_2 and 50 mL /L CO_2. The isometric response was recorded with an ink-writing recorder. After 2 h equilibration under 1 g-load, 50 μL Fructus Psoraleae (10, 20, 70, 200, 700, 1000 g/L) was added cumulatively into the tissue chamber in turn every 2 min to observe their effects on gallbladder muscle strips (cumulating final concentration of Fructus Psoraleae was 0.1, 0.3, 1.0, 3.0, 10.0, 20.0 g/L). The antagonists, including 4-DAMP, benzhydramine, hexamethonium, phentolamine, verapamil and idomethine were given 2 min before Fructus Psoraleae respectively to investigate the mechanisms involved.

RESULTS: Fructus Psoraleae dose-dependently increased the resting tension (r = 0.992, P < 0.001), decreased the mean contractile amplitude (r = 0.970, P < 0.001) and meanwhile increased the contractile frequency of the gallbladder muscle strip *in vitro* (r = 0.965, P < 0.001). The exciting action of Fructus Psoraleae on the resting tension could be partially blocked by 4-DAMP (the resting tension decreased from 1.37 ± 0.41 to 0.70 ± 0.35, P < 0.001), benzhydramine (from 1.37 ± 0.41 to 0.45 ± 0.38, P < 0.001), hexamethonium (from 1.37 ± 0.41 to 0.94 ± 0.23, P < 0.05), phentolamine (from 1.37 ± 0.41 to 0.89 ± 0.22, P < 0.01) and verapamil (from 1.37 ± 0.41 to 0.94 ± 0.26, P < 0.05). But the above antagonists had no significant effect on the action of Fructus Psoraleae–induced mean contractile amplitude (P > 0.05). Moreover, the increase of the contractile frequency due to Fructus Psoraleae was inhibited by 4-DAMP (decreased from 8.3 ± 1.2 to 6.8 ± 0.5, P < 0.01) and hexamethonium (from 8.3 ± 1.2 to 7.0 ± 0.9, P < 0.05). Idomethine had no significant effect on the Fructus Psoraleae-induced responses (P > 0.05).

CONCLUSION: Fructus Psoraleae enhances the motility of isolated gallbladder muscle strips from guinea pigs, in a dose-dependent manner. The effect of Fructus Psoraleae is partly related to M_3, N receptor, α receptor, H_1 receptor, Ca^{2+} channel, but not related to prostaglandin.

Key words: Fructus Psoraleae; Gallbladder smooth muscle strips; M_3, N, α, H_1 receptors; Ca^{2+} channel; Prostaglandin

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INTRODUCTION

Fructus Psoraleae is the dry fruit of the leguminous plant *Psoralea corylifolia L.* In recent years, researchers from home and abroad have made extensive studies on its chemical composition and extraction[1-4]. Studies on the pharmacological action of Fructus Psoraleae have been focused on its therapeutic effect on leucopenia, uterine bleeding, chronic bronchitis and psoriasis[5-8]. However, reports of the effect and mechanism of Fructus Psoraleae on the gallbladder smooth muscle strips *in vitro* are rare. In this experiment, we observed the effects of Fructus Psoraleae on the gallbladder muscle strips from guinea pigs and studied the possible mechanisms involved.

Table 1 Effect of Fructus Psoraleae on resting tension (g) of isolated gallbladder muscle strip after pretreated with antagonists (means ± SD)

Resting tension (g)	Fructus Psoraleae (g/L)						
	0	0.1	0.3	1	3	10	20
Pso	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.04	0.09 ± 0.04	0.24 ± 0.08[a]	0.88 ± 0.35[d]	1.37 ± 0.41[d]
Ben + Pso	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.04	0.07 ± 0.06	0.14 ± 0.08[c]	0.29 ± 0.17[b,h]	0.45 ± 0.38[d,h]
Phe + Pso	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.04	0.07 ± 0.07	0.17 ± 0.08[b]	0.46 ± 0.11[d,h]	0.89 ± 0.22[d,f]
4-DAMP + Pso	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.04[f]	0.09 ± 0.07[f]	0.30 ± 0.18[d,h]	0.70 ± 0.35[d,h]
Hex + Pso	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.08	0.16 ± 0.14[a]	0.42 ± 0.20[c,h]	0.94 ± 0.23[c,d]
Ido + Pso	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.02	0.09 ± 0.06	0.21 ± 0.12[a]	0.71 ± 0.23[d]	1.31 ± 0.41[d]
Iso + Pso	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.03[f]	0.10 ± 0.06[f]	0.38 ± 0.19[c,h]	0.94 ± 0.26[c,d]

[a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ *vs* control (under 1-g initial load the gallbladder spontaneous contraction when Fructus Psoraleae was 0 g/L). The resting tension of each strip in control was 0 ($n = 8$). [c]$P < 0.05$, [f]$P < 0.01$, [h]$P < 0.001$ *vs* Fructus Psoraleae (the resting tension of adding each concentration of Fructus Psoraleae) ($n = 8$). Pso: Fructus Psoraleae; Ben: Benzhydramine; Phe: Phentolamine; Hex: Hexamethonium; Ido: Idomethine; Iso: Verapamil.

MATERIALS AND METHODS

Materials

Fructus Psoraleae was ground into coarse powder, boiled, filtered and made into (1000 g/L) extract (the drug was prepared and tested by Gansu Institute for Drug Control), and then diluted to 10, 20, 70, 200, 700, 1000 g/L solutions respectively. The antagonists are as follows: 4-DAMP (1 μmol/L) (Sigma Chemicals Company), hexamethonium (10 μmol/L) (Sigma Chemicals Company), phentolamine (1 μmol/L) (Beijing No 13 Pharmaceutical Factory), verapamil (0.05 μmol/L) (Lanzhou Pharmaceutical Factory), idomethine (1 μmol/L)(Beijing Two-bridge Pharmaceutical Factory), and benzhydramine (1 μmol/L) (Jiangsu Taicang Pharmaceutical Factory). Guinea pigs of either sex, weighing between 350 and 450 g [purchased from Animal Center of Lanzhou Veterinary Institute, laboratory animal certificate FCXK (Gan2004-0005)]. The following equipments were used: JZ-BK external isometric force transducer (BK Company), LMS-ZB two channels recorder (Chengdu Equipment Factory, China).

Methods

Guinea pigs were fasted with free access to water for 24 h. They were hit on the head to become unconscious. The whole gallbladder was removed, quickly transferred to Krebs solution and rinsed. The wall of the gallbladder was incised from the end of the cystic duct to the base to make two or three longitudinal smooth muscle strips (8 mm × 3 mm). The mucosa was gently removed with forceps. Every longitudinal muscle strip was suspended in a tissue chamber which was continuously perfused with 5 mL Krebs solution (37℃), pH 7.4, and aerated with 950 mL/L O_2 and 50 mL /L CO_2. One end of the strip was fixed to a hook on the bottom of the chamber. The other end was connected to an external isometric force transducer (JZ-BK, BK). The strip was subjected to 1 g load tension and washed with 5 mL Krebs solution every 20 min. Motility of gallbladder strips in tissue chambers was simultaneously recorded by electrophysiolograph (LMS_ZB, Chengdu), including the resting tension, the mean contractile amplitude, and the contractile frequency[9]. After 2 h equilibration, 10, 20, 70, 200, 700, 1000 g/L Fructus Psoraleae was added cumulatively in turn every 2 min to observe their effects on contractility of gallbladder. The 4-DAMP (1 μmol/L), benzhydramine (1 μmol/L), hexamethonium (10 mol/L), phentolamine (1 μmol/L), verapamil (0.05 μmol/L) and idomethine (1 μmol/L) were added 2 min before Fructus Psoraleae was added to investigate whether the actions of Fructus Psoraleae were mediated *via* M_3, N receptor, α receptor, H_1 receptor, Ca^{2+} channel and prostaglandin(PG).

Statistical analysis

The data were presented as mean ± SD and analyzed with one-way ANOVA and correlation. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Fructus Psoraleae and antagonists plus Fructus Psoraleae on the resting tension of gallbladder muscle strips

Fructus Psoraleae dose-dependently increased the resting tension of gallbladder muscle strips *in vitro* ($r = 0.992$, $P < 0.001$). The 4-DAMP (1 μmol/L), benzhydramine (1 μmol/L), hexamethonium (10 μmol/L), phentolamine (1 μmol/L), verapamil (0.05 μmol/L) and idomethine (1 μmol/L) had no significant effects on the resting tension of gallbladder muscle strips. But when given 2 min before the administration of Fructus Psoraleae (0.1, 0.3, 1.0, 3.0, 10.0, 20.0 g/L), 4-DAMP, benzhydramine, hexamethonium, phentolamine and verapamil partly blocked the enhancing action of Fructus Psoraleae on the resting tension of gallbladder muscle strips. However, idomethine had no significant action on the increasing effect of Fructus Psoraleae on resting tension (Table 1).

Effect of Fructus Psoraleae and antagonists plus Fructus Psoraleae on the mean contractile amplitude of gallbladder muscle strips

Fructus Psoraleae dose-dependently decreased the mean contractile amplitude of gallbladder isolated smooth muscle strips ($r = 0.970$, $P < 0.001$). The 4-DAMP (1 μmol/L), benzhydramine (1 μmol/L), hexamethonium (10 μmol/L), phentolamine (1 μmol/L), verapamil (0.05 μmol/L) and idomethine (1 μmol/L) had no significant effects on the mean contractile amplitude of gallbladder muscle strips. When

Table 2 Effect of Fructus Psoraleae on the contractile amplitude (mm) of isolated gallbladder muscle strip after pretreated with antagonists (means ± SD)

Amplitude (mm)	Fructus psoraleae (g/L)						
	0	0.1	0.3	1	3	10	20
Pso	4.54 ± 0.64	4.23 ± 0.78	4.24 ± 0.84	4.07 ± 0.72	3.71 ± 0.59[a]	2.36 ± 0.77[d]	1.72 ± 0.74[d]
Ben + Pso	4.70 ± 0.57	4.43 ± 0.80	4.19 ± 0.88	4.09 ± 0.97	3.93 ± 0.97	3.55 ± 0.93[a]	2.37 ± 0.83[d]
Phe + Pso	4.71 ± 1.37	4.47 ± 1.42	4.64 ± 1.32	4.49 ± 1.35	4.17 ± 1.41	3.36 ± 1.43[a]	2.15 ± 0.65[d]
4-DAMP + Pso	4.91 ± 0.60	4.58 ± 0.71	4.23 ± 0.60	4.27 ± 0.84	3.84 ± 0.81[a]	3.70 ± 1.28[b]	2.34 ± 1.07[d]
Hex + Pso	5.48 ± 1.57	5.13 ± 1.64	5.34 ± 1.60	4.86 ± 1.63	4.80 ± 1.46	3.43 ± 1.10[b]	1.93 ± 1.02[d]
Ido + Pso	5.40 ± 1.30	5.01 ± 1.37	4.83 ± 1.33	4.62 ± 1.42	4.16 ± 1.29	3.39 ± 1.48[b]	2.24 ± 1.25[d]
Iso + Pso	4.47 ± 1.23	4.42 ± 1.37	4.60 ± 1.56	4.29 ± 1.32	4.00 ± 1.08	3.98 ± 1.74	2.80 ± 1.57[b]

[a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ *vs* control (under 1-g initial load the gallbladder spontaneous mean contraction amplitude when Fructus Psoraleae was 0 g/L) ($n = 8$).

Table 3 Effect of Fructus Psoraleae on the contractile frequency(waves/min) of isolated gallbladder muscle strip after pretreated with antagonists (means ± SD)

Frequency (w/min)	Fructus psoraleae (g/L)						
	0	0.1	0.3	1	3	10	20
Pso	3.3 ± 0.5	3.3 ± 0.7	3.3 ± 0.6	3.8 ± 0.9	4.6 ± 0.7[b]	7.3 ± 1.2[d]	8.3 ± 1.2[d]
Ben + Pso	3.4 ± 0.5	3.6 ± 0.4	3.6 ± 0.4	3.7 ± 0.4	4.6 ± 0.4[d]	6.3 ± 0.5[d]	7.3 ± 0.8[d]
Phe + Pso	3.5 ± 0.7	3.9 ± 0.8	3.9 ± 0.8	4.4 ± 1.0	5.1 ± 0.8[b]	7.1 ± 1.2[d]	9.1 ± 1.1[d]
4-DAMP + Pso	3.5 ± 0.4	3.5 ± 0.4	3.6 ± 0.6	3.9 ± 0.7	4.2 ± 0.5[a]	5.1 ± 0.9[d,h]	6.8 ± 0.5[d,f]
Hex + Pso	3.3 ± 0.5	3.5 ± 0.8	3.5 ± 0.5	3.9 ± 0.8	4.8 ± 1.0[d]	6.0 ± 0.7[c,d]	7.0 ± 0.9[c,d]
Ido + Pso	3.4 ± 0.5	3.4 ± 0.6	3.6 ± 0.7	3.8 ± 0.7	4.4 ± 0.7[a]	6.9 ± 1.4[d]	8.4 ± 1.1[d]
Iso + Pso	3.4 ± 0.5	3.4 ± 0.8	3.5 ± 0.8	3.8 ± 0.9	5.2 ± 1.4[b]	6.3 ± 1.2[d]	8.8 ± 1.6[d]

[a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ *vs* control (under 1-g initial load the gallbladder spontaneous contraction frequency when Fructus Psoraleae was 0 g/L) ($n = 8$). [c]$P < 0.05$, [f]$P < 0.01$, [h]$P < 0.001$ *vs* Fructus Psoraleae (contraction frequency of adding each concentration of Fructus Psoraleae) ($n = 8$).

added 2 min before administration of Fructus Psoraleae (0.1, 0.3, 1.0, 3.0, 10.0, 20.0 g/L), none of the above antagonists showed significant action on the decreasing effect of Fructus Psoraleae on the mean contractile amplitude (Table 2).

Effect of Fructus Psoraleae and antagonists plus Fructus Psoraleae on the contractile frequency of gallbladder muscle strips

Fructus Psoraleae dose-dependently increased the contractile frequency of gallbladder muscle strips from guinea pigs *in vitro* ($r = 0.965$, $P < 0.001$). The 4-DAMP (1 μmol/L), benzhydramine (1 μmol/L), hexamethonium (10 μmol/L), phentolamine (1 μmol/L), verapamil (0.05 μmol/L) and idomethine (1 μmol/L) had no significant effects on the contractile frequency of gallbladder muscle strips. When given 2 min before the administration of Fructus Psoraleae (0.1, 0.3, 1.0, 3.0, 10.0, 20.0 g/L), 4-DAMP and hexamethonium partly inhibited the action of Fructus Psoraleae on contractile frequency of gallbladder muscle strips; nevertheless, the other antagonists had no significant effects on the action of Fructus Psoraleae on the contractile frequency (Table 3).

DISCUSSION

Cholelithiasis is a common disease worldwide and many epidemiological studies have shown that the incidence of cholelithiasis has been on a rapid increase in some regions of the world since the last decade. Motor dysfunction of the gallbladder plays an important role in cholelithiasis and cholecystitis[10-12]. According to the literature, traditional Chinese medicine, western medicine and surgical management have been employed to treat cholelithiasis at home and abroad[13-16]. Herbal medicine is characterized by having few side effects and good curative effects, which is gradually accepted by people over the world. Zhou[17] reported that Fructus Psoraleae extract excited the intestinal canal *in vivo* and *in vitro*, and relaxed the uterus of guinea pigs. In the present study, we observed that Fructus Psoraleae significantly increased the resting tension and contractile frequency; meanwhile decreased the mean contractile amplitude of isolated gallbladder muscle strips of guinea pigs.

All smooth muscles in the gallbladder are involuntary and the nerves are controlled by both extrinsic and intrinsic nervous systems. Von Schrenck *et al*[18] reported that gallbladder smooth muscle cells possess muscarinic receptors of the M_3 type, which mediate contraction. Chen *et al*[19] reported the M_3 receptors are preferentially associated with the activation of phospholipase C, intracellular Ca^{2+} release and the calmodulin-dependent pathway. It has been identified that cholinergic N-receptor exists on the membrane of nerve ganglion cells of gallbladder smooth muscle. Our results showed that M_3

antagonist (4-DAMP) and hexamethonium(nicotinic cholinergic antagonist) partly blocked the enhancing action of Fructus Psoraleae on the resting tension and contractile frequency of gallbladder muscle strips, but not that of the contractile amplitude of the strips. These results suggested that Fructus Psoraleae excited gallbladder muscle strip *via* M_3 and ganglion N receptors.

Moreover, there were some relevant reports about histamine and histamine receptors. Jennings *et al*[20] proposed that histamine is distributed in the guinea-pig gallbladder and it could regulate contractile activity *via* activation of H_1 and H_2 but not H_3 receptor. Gallbladder muscle possesses stimulatory H_1 receptors and inhibitory H_2 receptors[21]. The depolarization and associated contraction of gallbladder smooth muscle represent the net effect of activation of both H (1) (excitatory) and H (2) (inhibitory) receptors, with the H (2) receptor-mediated response involving the activation of K (ATP) channels[22]. In this experiment, we observed that H_1 antagonist-benzhydramine partly inhibited the enhancing action of Fructus Psoraleae on the resting tension, but had no effect on the mean contractile amplitude and contractile frequency of gallbladder muscle strip. The results showed the excitatory action of Fructus Psoraleae on gallbladder muscle strip was possibly mediated *via* H_1 receptor. Yanaura *et al*[23] reported that contractions and relaxations produced by sympathomimetic amines are mediated by alpha-excitatory and beta-inhibitory adrenoceptors in the biliary system (gallbladder, common bile duct and sphinctor of Oddi) of guinea-pigs. We also observed that adnephrin antagonist-phentolamine partly inhibited the enhancing action of Fructus Psoraleae on the resting tension, but had no effect on the mean contractile amplitude and contractile frequency of gallbladder muscle strip, and the results revealed that the excitatory action of Fructus Psoraleae on gallbladder muscle strip was possibly mediated *via* α receptor. Ca^{2+}, which participates in excitation contraction coupling plays an important role in the contraction process of smooth muscle. The action potential in gallbladder smooth muscle (GBSM) is caused by Ca^{2+} entry through voltage-dependent Ca^{2+} channels (VDCC), which contributes to the GBSM contraction[24]. Shimada[25] believed the L-type Ca^{2+} current is dominant in gallbladder smooth muscle cells and may contribute to excitation-contraction coupling. In addition, our data showed that verapamil partly inhibited the exciting action of Fructus Psoraleae on the resting tension, suggesting that Fructus Psoraleae-induced gallbladder contraction was related to the Ca^{2+} channel. Verapamil is an L-type calcium channel blocker which inhibited the exciting effect induced by Fructus Psoraleae; whereas idomethine (prostaglandin enzyme suppressor) had no significant effects on the action of Fructus Psoraleae, indicating that the exciting action of Fructus Psoraleae on gallbladder smooth muscle strips was not related to prostaglandin (PG).

In summary, results from this study provide us new insights into the mechanisms underlying gallbladder motility and will be useful for further understanding of biliary dyskinesia diseases and the treatment.

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RAPID COMMUNICATION

K-19 mRNA RT-PCR in detecting micrometastasis in regional lymph nodes of gastric cancer

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Abstract

AIM: To investigate the value and prospect of RT-PCR in detecting micrometastasis in regional lymph nodes of gastric cancer.

METHODS: Histopathology was used and K19 mRNA expression was detected by RT-PCR in tumor tissues and lymph nodes from gastric cancer patients undergoing radical resection of gastric carcinoma.

RESULTS: K19 mRNA was expressed in all tumor specimens of 30 cases; of the 126 lymph nodes, 26 were histopathologically positive (20.6%), and 42 positive (33.3%) by RT-PCR. Amplification fragments of 460 and 540 bp were shown in all the tumor tissues and metastatic lymph nodes after K19 and β-actin RT-PCR, while only a 540 bp fragment appeared in the lymph nodes of non-tumor patients.

CONCLUSION: K19 mRNA RT-PCR is sensitive and specific in testing micrometastasis in regional lymph nodes of gastric cancer, and it is superior to routine histopathology.

Key words: K-19 mRNA; RT-PCR; Micrometastasis; Gastric cancer

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INTRODUCTION

Gastrointestinal cancer is the most common malignant tumor of the digestive tract. For histologically node-negative gastrointestinal cancer, even after curative resection of an early cancer, some patients die of metastasis and recurrence[1]. Metastasis and recurrence result from dissemination of cancer cells. Micrometastasis has been proposed by many investigators. Usually it is through blood and lymphatic vessels and no more than 2 mm in diameter[2]. Detection of occult metastatic cells is useful for prognosis, prediction of recurrence, and adjustment of therapies.

Among many prognostic factors, lymph node metastasis is one of the most useful indicators for patients with gastric and colorectal carcinoma[3-7]. Metastasis is usually detected by conventional histological examination, but many negative lymph nodes have micrometastasis on re-examination by serial sectioning and immunohistochemical assay[8-11]. Serial sectioning and immunohistochemical staining certainly increase the yield of occult metastasis, however, it seems to be time-consuming and labor intensive. These methods have not been performed routinely in most hospitals. To overcome this drawback, diagnostic procedures for the detection of micrometastasis at the genetic level have developed rapidly, such as RT-PCR[12-14]. RT-PCR can detect genes that are exclusively expressed in carcinoma cells but not in normal lymph nodes or bone marrow. It is a highly sensitive and specific method. It was reported that by RT-PCR it was possible to detect one cancer cell from among 10^4 to 10^6 normal appearing lymph node cells. Lymph node occult metastasis of gastrointestinal cancer indicated by K19 mRNA expression can be considered as confirmation of the presence of metastasis. The current study was designed to investigate the value and prospect of RT-PCR in detecting micrometastasis in regional lymph nodes of gastric cancer by examination of K19 mRNA expression.

MATERIALS AND METHODS

Tissue samples

The 30 tumor specimens and 126 lymph nodes were obtained through radical resection of gastric carcinoma of patients from the Department of General Surgery, First Hospital, Jilin city from 2001 to 2002, and tumor specimens were confirmed by pathology. The specimens

were processed immediately after the resection: tumor tissues were obtained; lymph nodes were peeled off carefully. Fat tissue and blood were wiped off, lymph nodes were cut into two halves by clean bistouries, and sterilized physiological saline was used for rinsing to prevent the contamination of tumor cells. One half of a lymph node was fixed by formaldehyde; the other half was immersed into liquid nitrogen, and then preserved in a -70℃ freezer till the next day for RNA extraction. Meanwhile, lymph nodes from 8 non-tumor patients were used as negative control.

Reagents

The reagents included TRI reagent (GIBCO), AMV, Taq enzyme, DNTPs and Rnasin (Promega), Marker and Olig (dt) (TaKaRa); the rest of the reagents were all homemade provided by local suppliers.

Methods

Primer design and synthesis: Primer design of CK19 and β-actin was based on previous methods[15] with some modifications. CK19 primer is: 5'-AGGTGGATTCCG CTCCGGGCA-3', 5'-ATCTTCCTGTCCCTCGAGC A-3'. The amplification fragment of the primer (Wubo Gene Corp., Beijing) was 460 bp. β-actin primer is: 5' -GTGGGGCCCCAGGCACCA-3', 5'-CTTCCTTAATG TCACGCACGATTTC-3'; the amplification fragment of the primer(Dinguo Bio Corp., Beijing) was 540 bp. It was used as an internal control to determine that the RNA did not decompose.

RNA extraction: TRIzol was used to extract total RNA. The absorbency (A) was tested. Then gel electrophoresis was performed to identify its components.

Reverse transcription (cRNA synthesis): Reverse transcription system was 50 μL, containing RNA 4 μL, Oligo(dt) 2 μL, 5×Buffer 10 μL, dNTPs 4 μL, Rnasin 1 μL, AMV 4 μL and DEPC-H_2O_2 5 μL incubated for 60 min at 42℃, to acquire cRNA.

PCR reaction: The reaction system was 100 μL, containing cDNA 25 μL, 10 × Buffer 10 μL, dNTPs 8 μL, K19 primer 2 μL, β-actin primer 0.5 μL, Taq enzyme 1 μL, $MgCl_2$ 6 μL, DEPC-H_2O_2 47.5 μL. The cycle parameters of the reaction system were 94℃ for 45 s, 55℃ for 45 s, 72℃ for 1 min, 35 cycles and extention for 10 min at 72℃.

PCR product analysis: PCR products were electrophoresed on 2% agarose gel, EB stained, and observed with ultraviolet light and photographed. The results were compared with pathological result.

Statistical analysis

We used t-test to compare between the histopathological results and RT-PCR results. $P < 0.05$ was taken as significant.

RESULTS

Comparison between histopathological result and K19 mRNA RT-PCR in detecting micrometastasis in regional lymph nodes

In 126 lymph nodes, 26 were positive in both routine histopathological testing and RT-PCR, K19 mRNA was expressed in 42 lymph nodes by RT-PCR amplification. It showed that there was metastasis in 16 lymph nodes which could not be found by histology examination. Of the regional lymph nodes, 20.6% were positive in histology, while 33.3% were positive in K19 mRNA by RT-PCR; and there were none that were positive for histology and negative by RT-PCR. In 30 cases of gastric tumor, 3 were positive in regional lymph nodes by RT-PCR while pathology showed no metastasis.

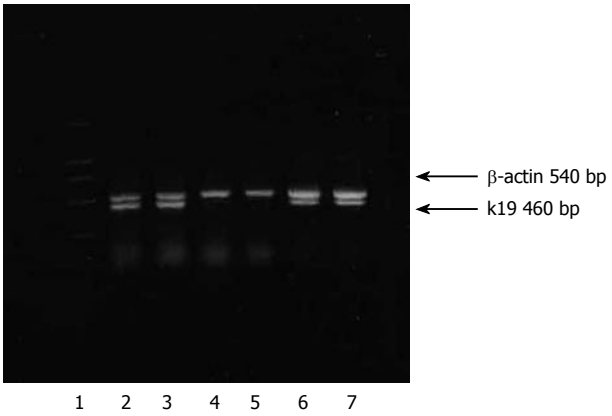

Figure 1 Representative results of RT-PCR. Lane 1: Marker; Lane 2: Tumor tissue sample; Lane 3: Negative lymph node by routine histopathology (K19 positive); Lane 4: Negative lymph node by routine histopathology (K19 negative); Lane 5: Normal lymph node of the non-tumor cases; Lanes 6, 7: Positive lymph node by routine histopathology.

Specificity of RT-PCR amplification

All the tumor tissues and metastatic lymph nodes showed amplification fragments of 460 bp and 540 bp after RT-PCR amplification of K19 mRNA and β-actin, while in the lymph nodes of 8 non-tumor cases it showed only specific amplification fragments of 540 bp, indicating no K19 mRNA amplification product was expressed. Thus this system had superior amplification specificity (Figure 1).

DISCUSSION

There has been no uniform criterion for micrometastasis. In general, a focus not larger than 2 mm is called micrometastasis, which can not be easily found by routine method, whereas RT-PCR could increase the detection rate greatly. Zheng *el al*[16] suggest selecting an ideal marker gene of the tumor as a histology specific marker. Keratin 19 is one of the histology markers, which is highly specific and only expressed in tumor tissue and tumor-originating normal tissue, but not expressed in normal mesenchymal tissues like lymph nodes[17-20].

In this study, K19 mRNA was expressed in both tumor tissue and metastatic lymph nodes, but not in non-tumor cases. It indicates that K19 mRNA is applicable to detect the micrometastasis in regional lymph nodes by RT-PCR amplification. Moreover, the results of our study suggest that RT-PCR is more sensitive than routine histopathology in detecting micrometastasis and K19 can be a sensitive index for detecting metastasis in regional lymph nodes,

which accords with Liu's report[21].

Because RT-PCR is the method that is sensitive and has great capability to amplify, sometimes there can be a pseudopositive, and the main reason could be that the lymph nodes are contaminated by tumor cells and normal epithelial cells and there is cross-contamination of byproduct during RT-PCR amplification. In the experiment, as the internal control, β-actin ensured reliability and the result showed that no K19 mRNA was expressed in any of the non-tumor cases under the same amplification condition, indicating that there was no pseudopositive in the RT-PCR amplification system. K19 mRNA RT-PCR has a high sensitivity and specificity in detecting micrometastasis in regional lymph nodes of gastric cancer and can detect the subtle metastasis which cannot be found by routine histology. This is of great clinical significance. According to the present staging criterion for gastroenteric cancer, the positive result will lead to change in the staging of tumor and alteration in therapy and prognosis judgment. Ye[22] and Yan[23] concluded that compared with lymph node-negative cases, K19 mRNA RT-PCR has obvious prognostic value on recurrence and survival time for the patients after the operation, even if there is a single metastasised tumor cell in the lymph node. Thus, the resection for early and intermediately staged patients should be as radical as possible[24-25], so as not to miss the micrometastasis in lymph nodes and to reduce the recurrence; and the adjuvant therapy and follow-up should be enhanced as well.

The development of the subtle tumor cells in the lymph nodes depends on the immunity of the human body and other factors, which is possible but does not develop into an obvious metastasis. At present, there are still some questions left unanswered about detecting the micrometastasis in regional lymph nodes: one is that pseudopositives are possible because of its great capability of amplification; the other is how to choose a more specific tumor marker. Before the advent of serial analysis of micrometastasis of tumor cells, tumor cells were separated by an immunomagnetic method and extracted, which was thought to be the most attractive technique[26]. Okadda[27] put forward that a multiple-marked RT-PCR has a better effect on detecting micrometastasis in lymph nodes, and researchers are trying to find more sensitive and specific tumor markers including combinations of multiple-markers.

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CASE REPORT

Corrosive injury to upper gastrointestinal tract: Still a major surgical dilemma

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Abstract

In the developed and developing countries, corrosive injury to the gastrointestinal system as a consequence of either accidental ingestion or as a result of self-harm has become a less common phenomenon compared to decades ago. This could partly be attributed to the tighter legislation imposed by the government in these countries on detergents and other corrosive products and general public awareness. Most busy upper gastrointestinal surgical units in these countries, especially in the developed countries will only encounter a small number of cases per year. Up to date knowledge on the best management approach is lacking. In this article, we present our experience of two contrasting cases of corrosive injury to the upper gastrointestinal tract in our thoracic unit in the last 2 years and an up-to-date Medline literature search has been carried out to highlight the areas of controversies in the management of corrosive injuries of the upper gastrointestinal tract. We concluded that the main principle in managing such patients requires a good understanding of the pathophysiology of corrosive injury in order to plan both acute and future management. Each patient must be evaluated individually as the clinical picture varies widely. Signs and symptoms alone are an unreliable guide to injury.

Key words: Acid; Alkali; Oesophageal stricture; Endoscopy; Steroids; Oesophageal and gastric carcinoma

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INTRODUCTION

In the developed and developing countries, corrosive injury to the gastrointestinal system as a consequence of either accidental ingestion or as a result of self-harm has become less of a common phenomenon. This could partly be attributed to the tighter legislation imposed by the government on detergents and other corrosive products and general public awareness[1,2]. Most busy upper gastrointestinal (GIT) surgical units, especially in the developed countries will only encounter a small number of cases per year. Up to date knowledge on the best management approach may therefore be lacking.

We present two contrasting cases of corrosive injury to the upper GIT, which presented to our thoracic unit in the last 2 years to highlight the contrasting aspects of chemical burn injury to the upper GIT. A Medline search has been carried out to extract relevant articles to enable us to perform a literature review to discuss the areas of controversies in the management of corrosive injuries of the upper GIT.

CASE REPORTS

Case 1

A 22-year-old male with learning disability attended the Casualty Department following accidental ingestion of a cupful of 30% caustic soda and had vomited immediately after it. On presentation, his voice was hoarse. He was also short of breath and drooling his saliva. On examination, he had a red, swollen tongue and his oropharynx was oedematous and inflamed. He was intubated to secure his airway and transferred to the intensive care unit. Other supportive treatments received included intravenous proton pump inhibitor (PPI) and total parental nutrition (TPN). He was extubated 2 d later. Early oesophagogastroscopy (Figure 1) revealed generally inflamed oropharynx and Savary grade 3 oesophagitis from 20 cm. Examination beyond this point was not attempted. Barium meal (Figure 2) carried out two weeks later showed a long stricture segment from just distal to the hypopharynx to the oesophago-gastric junction. The patient did not receive steroid therapy during any stage of his treatment. He was successfully managed with repeated progressively time spaced dilatation using a guide wire under fluoroscopy. He currently attends Day Procedure Unit every 6-12 wk for oesophageal dilatation.

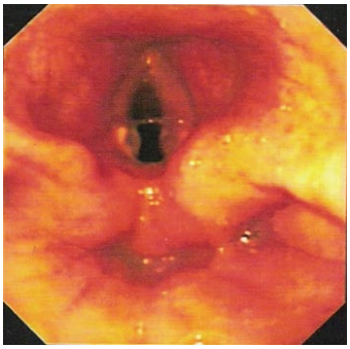

Figure 1 Endoscopic picture showing gross laryngeal edema with inflamed adjacent structures (case 1).

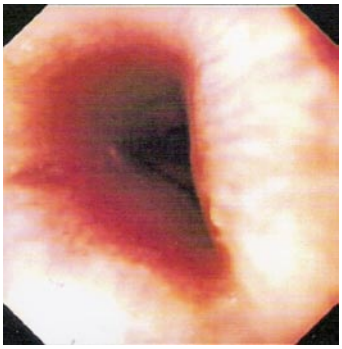

Figure 3 Oesophagogastric dissociation (OGD) of the patient after ingestion of battery acid showing circumferential burn to the lower oesophagus (case 2).

Figure 2 Barium meal showing a long stricture segment from just distal to the hypopharynx to the oesophago-gastric junction (case 1).

Figure 4 Barium study of patient showing partial gastric outlet obstruction with marked gastric dilatation with an irregular stricture of the pylorus and proximal duodenum (case 2).

Case 2

A 33-year-old male with a history of overdose and assaults presented to the Casualty Department with hoarseness and stridor following ingestion of about 40 mL of battery acid (hydrochloric acid) secondary to deliberate self-harm. He was intubated to secure his airway and transferred to ITU. A CT scan of his chest and abdomen showed thickened distal oesophagus and stomach, small bilateral pleural effusion and no obvious sign of perforation. Early endoscopy showed inflammation and ulceration of the pharynx and oesophagus with contact bleeding and circumferential ulceration of the oesophageal mucosa at 25 cm (Figure 3). His stomach was filled with blood. The immediate supportive treatments included intravenous PPI, TPN for nutrition, steroids and broad-spectrum antibiotics because of gross laryngeal oedema and positive blood culture. He improved on conservative management and following extubation was allowed oral feeding. Endoscopy was repeated 10 d later and it showed small ulcers at the level of the vocal cords. Upper oesophagus was relatively spared. A tight cricopharyngeus was noted and his lower oesophagus showed a circumferential burn with slough. Similar findings were noted on the mid-body of the stomach and the antrum but the duodenum was spared.

He was discharged home three weeks later only to be readmitted a week and a half post-discharge with symptoms and signs of gastric outlet obstruction. Endoscopy at this stage showed a normal oesophagus with an ulcerated and scarred gastric pylorus. The endoscope failed to advance beyond this point. These findings were confirmed on a dilute barium swallow (Figure 4). Roux-en-Y gastrojejunostomy was performed subsequently in order to bypass the stricture. He remained well post-operatively.

DISCUSSION

There are a vast variety of chemicals commonly available in a modern western household that can be ingested either inadvertently or intentionally. Failure to recognize the seriousness of the accident and to provide adequate therapy could result in serious morbidity and mortality. Children account for more than 80% of accidental corrosive ingestion but ingestion in adult is more often of suicidal intent, and, therefore, tend to be more serious[3]. The mortality rate is between 10% to 20% and rises to 78% in cases of attempted suicide[4]. The extent of the injury depends on the type of agent, its concentration, quantity and physical state, the duration of exposure and the presence of food particles in the stomach[5-7].

Pathophysiology

The dichotomy of oesophageal versus gastric injury in cases of acid and alkali ingestion has long been recognized by surgeons and gastroenterologists[8]. Whilst acid is said to "lick the oesophagus and bite the pyloric antrum", alkaline tends to cause a more uniformly severe mucosal injury to the oesophagus[3,6,9]. Although acid injury is usually limited to the stomach, 6%-20% of patients have other associated oesophageal and small intestinal injuries[6]. Our two cases clearly illustrated this with the caustic injury in case 1 causing extensive oesophageal injury whilst the acid resulting in gastric injury and sparing the oesophagus.

Acid injuries cause "coagulation necrosis" on tissue contact; the coagulum formed hinders any further tissue

penetration[3,4,9,10]. On the other hand, caustic injuries induce "liquefaction necrosis", a process that leads to the dissolution of protein and collagen, saponification of fats, dehydration of tissues and thrombosis of blood vessels, resulting in deeper tissue injury[3,4,9,10]. Zargar *et al* noted that acute gastric injury was present in 85.4% of their patients who had ingested acid, involving mainly the distal half of the stomach with 44.4% having late complications in the form of pyloric or antral stenosis and linitis plastica-like deformity[11]. The relative sparing of the duodenum is thought to be due to pyloric spasm induced by the irritant acid in the antrum and the alkaline pH of the duodenum[11]. Our patient who ingested battery acid developed partial gastric outlet obstruction. Contrast study performed documented duodenal rigidity and lack of normal mucosal pattern. However, the distinction between the expected sites of gastrointestinal injury following acid versus alkali ingestion is not always clear.

Burn classification

Injuries secondary to chemical burns of the upper gastrointestinal tract are classified in similar fashion to thermal burn of the skin. They are classified into three degrees based only on the extent and severity of the superficial lesions[12]. An appreciation of the depth of the involvement may improve our treatment, but at present, no definite measurements of the depth can be made, and the grading at best is subjective. Endoscopic ultrasound may provide an answer. A third-degree burn can easily be mistaken for a second-degree burn[9].

Oropharyngeal burns and clinical symptoms have a low predictive value for severity of oesophageal injury[13,14]. Haller *et al* observed that 70% of their patients with oropharyngeal burns did not have significant damage to the oesophagus[15]. Both our patients had severe oropharyngeal burns requiring immediate intubation in order to protect their airway but only one developed severe oesophageal injury.

Early versus late endoscopy

Early endoscopy is regarded as the most appropriate measure based on which clinical decisions are made in people who have ingested corrosive substances[3,8,12,16-18]. The majority of physicians and surgeons now favour early endoscopy. Nevertheless, early endoscopy in the hands of a less-experienced endoscopist could be hazardous[2,10]. It is important to minimize force and air insufflation when passing the endoscope in this group of patients[10]. The risk of oesophageal perforation is low if the procedure is performed under general anaesthesia and the endoscope is passed to the first burned area but not beyond it[16]. Hawkins *et al* recommend diagnostic oesophagoscopy under general anaesthesia within the first 36 h of corrosive ingestion. In severe oropharyngeal burns, endoscopy may be deferred up to seven days to allow the acute oedema to subside, thereby reducing the risk of airway complication[8]. Zargar *et al* performed endoscopies on 88 patients within 96 h following corrosive ingestion and found no complications related to the procedure[19]. Others have also documented the safety of early endoscopy[3]. Both our patients had endoscopy carried out within 72 h following corrosive ingestion. Early endoscopy is essential in the continuing management of patients with corrosive injury as it affords an opportunity to verify directly the healing state of the mucosa and may be of value in predicting which patients require further early intervention[1,2,8,19,20].

Endoscopy is not without its limitations[1]. It is difficult to assess the depth of any burn with absolute certainty by observing superficial epithelial necrosis[2]. If a severe burn is encountered in the upper third of the oesophagus, the scope is not passed beyond this point. In this case, it will be difficult to ascertain the degree of injury to the rest of the oesophagus[3]. The area of burn may not be visualised, thus delaying the diagnosis[2]. Others have attempted the use of endoscopic ultrasound to improve the accuracy of diagnosis. However, Chiu *et al* did not find concomitant use of endoscopic ultrasound (EUS) useful in improving the accuracy of predicting early or late complication of stricture[21].

Manometric study

Genc and Mutaf investigated the use of manometric studies and suggested that it could give important data about the severity of the initial oesophageal injury[22]. Dantas *et al* showed that a majority of their patients with caustic oesophageal injury exhibit alterations in oesophageal motility, ranging from low amplitude non-peristaltic contractions to some degree of alteration of lower oesophageal sphincter pressure[5]. Thus, it could play a role in determining the prognosis. PPI is generally prescribed on the basis of associated reflux. This data provide supporting evidence for its use.

Complications of corrosive ingestion

Severe complications, often life threatening are common following corrosive injury to the upper gastrointestinal tract. These include tracheobronchial fistula in 3%, severe haemorrhage secondary to gastric involvement, aortoenteric fistula or gastrocolic fistula, strictures and perforation in 10% of cases[3,5,13,20]. Stricture formation, by far, remains the main long-term complication of this injury. Over 90% of patients with third-degree burns go on to develop stricture and 15%-30% if they have second-degree burns [3]. Mamede *et al* observed an 89.3% incidence of oesophagitis in their 37-year historical series; 72.6% of the cases involved progression to stenosis and 1% died during acute phase[7]. A lumen >10 mm in diameter is thought not to impede normal life and should be left alone[13].

Early use of steroids and antibiotic: Prevention of stricture formation

Corticosteroids inhibit the transcription of certain matrix protein genes, procollagen, fibronectin, TGF-β and many cytokines. They also reduce the synthesis of α2-macroglobulin, an inhibitor of collagenase activity[23]. Animal experiments have shown that if antibiotics and steroids are given early following ingestion of a corrosive substance, the likelihood of stricture formation is reduced[2,24]. Bautista *et al* found dexamethasone more

effective than prednisolone in preventing stricture formation following experimental oesophageal burns[24]. Mamade *et al* concluded from their clinical experience of 239 cases over 38 years that lower doses of steroid have little effect on the prophylaxis of stricture[25]. Higher doses only seem to contribute to the onset of complications such as increased vulnerability to infection and gastrointestinal bleeding[16,25]. Several authors have found corticosteroids ineffective in preventing oesophageal stricture[10,12,14,16,18]. This has also been shown in a more recent randomised controlled clinical trial in children.

Intra-lesional corticosteroid therapy has shown beneficial effects for refractory oesophageal strictures caused by corrosive burns. A report by Kochhar *et al* concluded that patients treated in this way experience a longer dilatation-free interval, thus requiring fewer dilations[26]. However, the number of patients involved in the study is small.

In our two contrasting cases, steroid was given to the patient with acid burns for the first 24 h in view of the severity of laryngeal oedema at presentation to avoid casualty. In another patient who suffered from caustic burns, steroid was not given because the literature suggests that the complication risk outweighs the efficacy in preventing stricture formation.

To date, there is no convincing evidence supporting the use of antibiotics in reducing stricture formation[18]. An animal study has shown that it could decrease infection in steroid treated burns[3]. Kirsh *et al* recommended the use of antibiotics for 7 d to 2 wk as a means to both decrease the risk of pulmonary infection and bacterial invasion through the injured oesophagus into the mediastinum[2]. Our general consensus when treating a patient with such injury is that antibiotic treatment should only be commenced when the patient is treated with steroids or there are signs of infection with source of infection and infecting organism identified. Prophylactic use of antibiotics without steroid treatment is unjustified[18].

Routine use of nasogastric (NG) tube

Mamede *et al* reported a significant lower incidence of stricture formation with routine use of NG tube for 15 d following the injury[25]. However the NG tube could not act as an oesophageal 'mould' because one could expect the stenosing effect to continue longer than 15 d. Wijburg *et al* also reported a decline in stricture formation in a patient with long-term nasogastric tube placement[27].

However, contrasting results were obtained from other literatures stating that long-term indwelling nasogastric insertion is known to cause long strictures of the oesophagus even in patients without oesophageal burns[3,18]. We do not advocate the use of a NG tube as we have experienced a number of patients who developed complex stricture following nasogastric insertion. Furthermore, the presence of a NG tube will aggravate reflux by making the lower oesophageal sphincter incompetent. We used TPN in both patients and would have proceeded to feeding jejunostomy if oral feeding was not soon established.

Experimental studies to prevent stenosis

In a recent experimental study, cytokines have been used successfully in preventing stricture formation by Berthet and colleagues[20]. The theory was based on the rationale of the inflammatory process and cascade of events. Epidermal growth factor (EGF) was used because of its properties of fibroblast stimulation and improvement of local vascular conditions. Interferon-γ (IFN-γ) was also used to reduce fibrosis as it inhibits collagen I and III formation and fibronectin synthesis[20]. Hydroxyproline was used as an indirect measurement of collagen production as it is the ultimate product of collagen degradation[20]. Stenosis was not observed in treated animals[20]. There was a lower level of hydroxyproline in combined treatment compared to EGF alone[20].

Kaygusuz *et al* investigated the effect of interferon-α-2b and octreotide in the treatment of corrosive burns of the oesophagus[28]. A histopathological examination of the exposed oesophagus demonstrated that octreotide and interferon-α-2b distinctively depressed the fibrotic activity in the second phase of wound healing that occurred in the oesophageal wall after a corrosive burn[28]. Gunel *et al* showed in their animal experiment that treatment with an antioxidant, such as vitamin E and methylprednisolone decreased tissue hydroxyproline and thus, inhibiting new collagen synthesis and stricture formation following corrosive injury[29]. However, all these studies are only carried out on animals and these treatments have not been tested on humans.

Management

The acute management is based on the acute trauma life support (ATLS) guidelines for burn injury. This includes securing the airway, pain relief and attending to adequate intravenous fluid replacement. Tracheostomy may be necessary in cases of severe laryngeal oedema, whereby tracheal intubation fails and there is a danger of completely closing over of the airway due to the edema[2,30]. The aim of treatment at this stage is to stabilize vital parameters. The patient is kept strictly nil by mouth in acute phase. A plain chest radiograph is advisable and might reveal signs of perforation, i.e. pneumomediastinum and free air under the diaphragm[3,19,28,30]. However the sensitivity is low and if perforation is suspected, diluted barium swallow should be carried out. It is crucial that the attending medical officers are aware of the severity of such injury and able to identify life-threatening complications associated with the injury. The use of antidote such as water or milk does not seem to prevent stenosis[25]. Endoscopy is the diagnostic procedure of choice in the absence of known perforation[3]. Patients with perforation require immediate surgery[3]. Gastric acid suppression with PPIs and H_2-antagonists are often used in corrosive burn injury as oesophagitis and gastritis are common and patients have been kept fasting[18]. This treatment has been employed in both our patients in order to suppress gastric acid production and to prevent stress ulcers in the stomach.

Our first patient with the alkaline burns of the gastrointestinal tract who later developed oesophageal strictures was managed with frequently repeated dilatation. Hawkins *et al* reported a relatively high success rate with dilatation[16]. Dilatation could be antegrade or retrograde or a combination of

both[1]. Early dilatation is not recommended due to associated high incidence of perforation and associated morbidity[3]. Most authors advised waiting 3 to 6 wk after the initial injury before attempting oesophageal dilatation[3,30]. Overall, oesophageal dilatation has proved to give good results in short strictures but might be dangerous for long and narrow oesophageal strictures[14,20]. Complex strictures are refractory to dilation therapy and fluoroscopic guidance has a valuable role in managing these types of strictures[23]. Repeat dilation sessions are needed in most cases with a goal of achieving a luminal diameter of 12 mm or larger in order to alleviate symptoms of solid dysphagia[23]. We suspect that the strategy of intense PPI therapy and repeated dilatation will reduce the number of impassable stricture that otherwise would have required oesophageal resection and reconstructive surgery.

The second patient with acid burns of the gastrointestinal tract developed gastric outlet obstruction within 3 wk of injury. The use of a steroid is of questionable value, and overall evidence from the literatures is not in favour of routine use. This is because it could mask the clinical signs of free perforation and infection[12,13,16]. Therefore, its use is limited mainly to patients with severe laryngeal oedema. Antibiotics have been used in this case for the treatment of an obvious chest infection. Gastric outlet obstruction has been found in association with oesophageal stricture in the region in 20% of cases[17]. In some cases gastric outlet obstruction can yield balloon dilatation but our patient required surgical bypass because of the complex nature of his stenosis. Alternative surgical reconstruction would be hemigastrectomy and resection of the first part of the duodenum with Bilroth I reconstruction. At the time of presentation, our patient was unfit for such a major operation.

Understanding the pathophysiology of corrosive injury is important in planning both acute and on-going management. Scar retraction begins as early as the end of the second week and lasts for 6 mo. Six to twelve months is considered the average time before full fibrosis is achieved after the injury[31]. Oesophagectomy carried out too early prior to the scar tissue maturation might increase the risk of anastomostic stenosis[32]. Han *et al* advocate delaying major reconstructive surgery in patients with caustic burns for at least 6 mo from time of injury provided that emergency surgery is not indicated[32]. Emergency oesophagectomy plus exteriorisation or immediate reconstruction is however indicated in cases of perforation and contamination of the mediastinum[9].

Risk of carcinoma

The association of lye stricture and carcinoma of the oesophagus has been known since 1896[2]. Kiviranta believed that the incidence of oesophageal cancer among victims of lye stricture is at least 1000 times greater than that in the normal population[33]. The interval between lye ingestion and the development of carcinoma ranges between 25 to 40 years. However, this risk may be overestimated. Marchand did not encounter a single case in 135 patients with caustic strictures of the oesophagus over a period of 6 years[34]. Carver and colleague had 2 patients out of 233 patients with lye strictures over a period of 25 years[35]. Mamede and colleague found 4 (1.6%) out of the 239 patients from their 37-year historical series developed oesophageal cancer after caustic soda ingestion[7]. In these cases, operative risk may exceed the potential risk of cancer.

The risk of gastric cancer is less known[8]. Gray and Holmes first reported in 1948 findings of squamous metaplasia in the stomach of a patient who had ingested acid[36]. Similar findings were subsequently reported by O'Donnell and colleagues[37] and later by Eaton and Tennekoon[38]. Some surgeons are more aggressive in resection of the involved stomach because of the danger of subsequent gastric metaplasia[6,9]. The predisposition to cancer justifies regular follow-up and surveillance endoscopy. However, the patient should be warned of the cumulative dangers of other risk factors for oesophageal cancer, such as alcohol abuse and smoking[30].

CONCLUSION

The literature on treatment of patients with corrosive injuries to the upper gastrointestinal tract is both controversial and inconclusive. The main principle in managing such patients is that each patient must be evaluated individually as the clinical picture varies widely. Signs and symptoms alone are an unreliable guide to injury. Both the acute and the chronic phases of the clinical presentation require different management. Psychiatric support is sometimes needed during both the acute and chronic phases. The general consensus is that the initial treatment is supportive; ensuring the airway is patent and to establish haemodynamic stability. Early endoscopy has a crucial role in both diagnosing the severity of the injury, as well as, in managing the patient. Total parenteral nutrition is a useful adjunct. Operation is generally reserved for patients who have ingested large amounts of corrosive substance and in whom tissue necrosis is highly likely. Extensive necrosis noted on endoscopy and patients with evidence of perforation are indications for immediate surgical intervention. As for intractable oesophageal strictures when dilatation is dangerous or impossible, surgical intervention may be unavoidable. However, this must be balanced against the mortality and sometimes considerable morbidity following surgery[2,30]. Follow up endoscopy should be carried out within 6 wk following discharge from the hospital[30]. Diligent follow-up is advised to ensure patient has satisfactory gastrointestinal function restored and to correct late onset complications.

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CASE REPORT

Enteric neuropathology of congenital intestinal obstruction: A case report

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Abstract

Experimental evidence indicates that chronic mechanical sub-occlusion of the intestine may damage the enteric nervous system (ENS), although data in humans are lacking. We here describe the first case of enteric degenerative neuropathy related to a congenital obstruction of the gut. A 3-year and 9-mo old girl began to complain of vomiting, abdominal distension, constipation with air-fluid levels at plane abdominal radiology.

Her subsequent medical history was characterized by 3 operations: the first showed dilated duodeno-jejunal loops in the absence of occlusive lesions; the second (2 years later) was performed to obtain full-thickness biopsies of the dilated intestinal loops and revealed hyperganglionosis at histopathology; the third (9 years after the hyperganglionosis was identified) disclosed a Ladd's band which was removed and the associated gut malrotation was corrected. Repeated intraoperative full-thickness biopsies showed enteric degenerative neuropathy along with reduced interstitial cells of Cajal network in dilated loops above the obstruction and a normal neuromuscular layer below the Ladd's band.

One year after the latest surgery the patient tolerated oral feeding and did well, suggesting that congenital (partial) mechanical obstruction of the small bowel in humans can evoke progressive adaptive changes of the ENS which are similar to those found in animal models of intestinal mechanical occlusion. Such ENS changes mimic neuronal abnormalities observed in intestinal pseudo-obstruction.

Key words: Enteric neuropathy; Chronic intestinal pseudo-obstruction; Congenital intestinal obstruction; Ladd's band; Enteric nervous system

Di Nardo G, Stanghellini V, Cucchiara S, Barbara G, Pasquinelli G, Santini D, Felicani C, Grazi G, Pinna AD, Cogliandro R, Cremon C, Gori A, Corinaldesi R, Sanders KM, De Giorgio R. Enteric neuropathology of congenital intestinal obstruction: A case report. *World J Gastroenterol* 2006; 12(32): 5229-5233

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INTRODUCTION

The enteric nervous system (ENS), the third component of the autonomic nervous system, plays a crucial role in the control of gastrointestinal functions, including motility, secretion, absorption, blood flow, mucosal growth and aspects of the local immune system[1]. Hence, any condition altering the integrity of the ENS is responsible for a wide spectrum of disorders affecting the gastrointestinal tract. Abnormalities of the ENS (also referred to as enteric neuropathies) may be secondary to a variety of inflammatory, infectious, metabolic and neurological diseases, or they can be labelled idiopathic when no causes can be found[2-6]. The most common clinical manifestation related to an underlying enteric neuropathy (either idiopathic or secondary) is a severe functional impairment of gastrointestinal motility as that identifiable in patients with chronic intestinal pseudo-obstruction (CIPO)[4,7,8]. The diagnosis of CIPO is based on the exclusion of any mechanical lesion occluding the

gut lumen[4,7,8]. On the other hand, in animals chronic incomplete mechanical occlusion of the intestine may induce ENS damage[5]. The effects of chronic obstruction on the human ENS are still unknown.

Herein we describe the case of a girl in whom an initial diagnostic work-up suggested a diagnosis of CIPO with an underlying neuropathy characterized by hyperganglionosis of the ENS, whereas a laparotomy about 10 years later showed a chronic congenital obstruction of the small bowel due to a Ladd's band. Full-thickness gut biopsies, taken during this last laparotomy, showed an enteric degenerative neuropathy in the bowel loops located above the obstruction. Because the correct pathology remained undiagnosed for a long time, this case can be considered a human model of mechanical partial obstruction of the gut. Specifically the case indicates that a long-standing ("chronic") intestinal sub-occlusion can evoke progressive changes of the ENS similar to those found in animal models, and that the observed ENS changes mimic abnormalities often found in CIPO related to idiopathic neuropathies.

CASE REPORT

G. S. was a full term baby girl weighing 2040 g at delivery whose clinical history was unremarkable until 3 years and 9 mo of age when she began to complain of post-prandial vomiting (often bilious) and constipation. Clinical examination revealed abdominal distension, dehydration and decreased bowel sounds. Systemic and neurologic diseases, as well as infections and malabsorption syndromes were excluded by physical examination and blood tests. X-ray of the small bowel showed mild gaseous distension of the proximal small bowel loops with air-fluid levels although a CT scan failed to reveal any mechanical obstruction. Due to the severity of the clinical picture and the marked small bowel distension, the patient underwent laparoscopy showing duodeno-jejunal dilatation with no obvious mechanical cause. The patient was discharged with a diagnosis of intestinal pseudo-obstruction and treated with prokinetics and a low-fiber diet.

Subsequently, she had recurrent episodes of vomiting, abdominal pain, early satiety and fullness. Because of the persistence of severe dyspeptic symptoms, at 5 years and 7 mo of age the patient was referred to a tertiary center for pediatric gastroenterology where, based on the clinical history and previous examinations, a decision to undertake an explorative laparotomy was reached. The intra-operative evaluation showed a marked degree of duodeno-jejunal dilatation but no mechanical obstruction.

Analysis of full-thickness biopsies taken from the dilated jejunal loop revealed enlarged myenteric and submucosal neurons whose number was increased as compared to sex-age-matched normal controls[9]. A further important feature was the increased density of the nerve fibers in the lamina propria and submucosa. Based on the histopathology suggesting a case of hyperganglionosis, the diagnosis of CIPO was confirmed and the patient received several courses of prokinetics, metronidazole and high-caloric liquid diet supplementation.

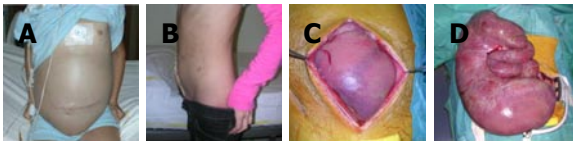

Figure 1 Representative pictures showing clinical (**A** and **B**) and laparotomic views (**C** and **D**) observed in this case. Note (**A**) the hugely distended abdomen and (**C** and **D**) abnormally dilated loops located proximally to the removed Ladd's band (not visible in these examples) at laparotomy. B illustrates the considerable deflation of the abdomen following surgery.

During a subsequent recovery the patient underwent an upper gastro-intestinal manometry which showed a pattern indicative of neurogenic-type CIPO. Due to the progressive worsening of her clinical status, at 14 years and 2 mo of age, the patient was referred to our center in Bologna. Physical examination showed a highly distended (Figure 1A) and painful abdomen, with absence of peristaltic sounds. An X-ray examination showed multiple air-fluid levels in the upper abdomen with a striking elevation of the diaphragm. In order to achieve bowel decompression, she underwent surgery. At laparotomy a Ladd's band was detected and removed with correction of the associated malrotation. Bowel loops with marked dilatation (Figures 1C and 1D) were resected and a protective ileostomy was created. Full-thickness biopsies were taken from the loops located proximally (dilated segments) and distally (macroscopically normal) to the Ladd's band.

Following surgery, the clinical course was uneventful with immediate deflation of the abdomen. One year after the last laparotomy the patient was healthy and tolerated oral feeding, her height and weight were markedly increased, and her quality of life was good (Figure 1B).

Immunohistochemical analysis

Full-thickness tissue specimens were processed for immunohistochemistry according to standard protocols commonly applied in our laboratory. Compared to controls (jejunal specimens collected from patients operated on for intestinal bleeding due to angiodysplasia; $n = 4$, 2 females, age range: 6-16 years) (Figure 2A) and the non-dilated segment (Figure 2B), the immunohistochemical evaluation of biopsies taken from the dilated loop showed evidence of intrinsic neuropathy of the gastrointestinal tract characterized by severe myenteric and submucosal neuron depletion (Figure 2C), as identified by the reduced number of neural elements labeled by the general marker neuron specific enolase (NSE) (purchased from DakoCytomation, Milan, Italy). Furthermore, analysis of several transmitters/neuromodulators of the ENS demonstrated a marked decrease of substance P, vasoactive intestinal polypeptide, calcitonin gene-related peptide (all these antibodies were kindly donated by Dr. C. Sternini and H. E. Wong, Center for Ulcer Research and Education/Digestive Diseases Center, UCLA School of Medicine, Los Angeles, CA) and nitric oxide synthase (purchased from BD Biosciences, San Jose, CA) in the myenteric and submucosal neurons and related processes of the dilated segment compared to

the non-dilated segment and controls. In order to explore the possible abnormalities of neuronal cell survival, we used a specific antibody against the product of B-cell lymphoma-2 (*BCL-2*) (DakoCytomation, Milan, Italy), a gene encoding a protein involved in cellular pathways of neuronal apoptosis[10,11]. Compared to the controls (Figure 2D) and the non-dilated segment (Figure 2E), BCL-2 immunoreactivity of the dilated segment was markedly reduced in myenteric (Figure 2F) and submucosal neurons and nerve processes. The interstitial cells of Cajal (ICC), visualized by an antibody against c-Kit (DakoCytomation, Milan, Italy), were markedly decreased in the dilated segment compared to the non-dilated segment and controls. Compared to the controls, the anti-α-smooth muscle actin antibody (DakoCytomation, Milan, Italy) showed an apparently normal muscular layer both in dilated and in non-dilated segments.

Transmission electron microscopy study

Full-thickness tissue specimens were also processed for electron microscopy according to standard protocols commonly applied in our laboratory.

Compared to the controls (Figure 2G) and the non-dilated segment (Figure 2H), electron microscopy analysis of myenteric (Figure 2I) and submucosal neurons of the dilated loop showed degenerative features characterized by nuclear chromatin clumping, shrinkage of the cell body and cytoplasmic vacuoles mostly deriving from the enlargement and matrix clearing of mitochondria.

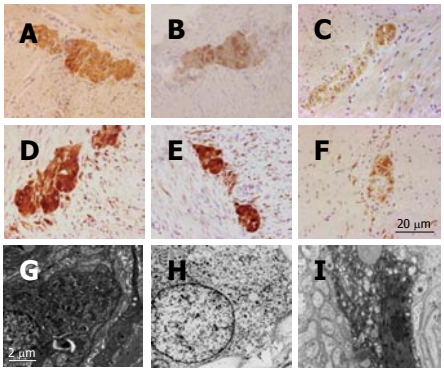

Figure 2 Representative examples illustrating the general neuronal marker NSE (**A**, **B**, **C**) and BCL-2 (**D**, **E**, **F**) immunoreactivities in the neuromuscular layer of a control subject (**A**, **D**), in the non dilated loop distal to the congenital obstruction of the patient (**B**, **E**) and in the dilated loop proximal to the congenital obstruction of the patient (**C**, **F**). Note the marked reduction of NSE and BCL-2 immunoreactivities in myenteric ganglion cells and nerve fibers targeting the muscular layer observed only in the dilated loop. Streptavidin biotin immunoperoxidase technique. *Calibration bar* (**A-F**): 20 μm. Compared to controls (**G**) and the non-dilated segment (**H**), myenteric neurons of the present case (representative example in **I**) showed chromatin clumping, cell body shrinkage and cytoplasmic vacuolization. Uranyl acetate and lead citrate staining, transmission electron microscopy. *Calibration bar* (**G**, **H**, **I**): 2 μm.

DISCUSSION

The present case illustrates the occurrence of gut failure as a result of longstanding mechanical small bowel obstruction due to a Ladd's band, which has been unrecognized for more than 10 years despite extensive radiological investigation and 3 explorative laparotomies. A Ladd's band, which arises from the posterior abdominal peritoneum and extends from the liver to the colon (passing anteriorly to the duodenum), is responsible for gut malrotation[12]. Symptomatic patients usually present either acutely with bowel obstruction and intestinal ischemia, or chronically with vague abdominal pain. Chronic symptoms can often make diagnosis difficult, as was the case in our patient. Although Ladd's band and associated gut malrotation are more common in the first 2 wk of life[13,14], our case provides evidence that patients having this developmental abnormality may be found in childhood.

The clinical and laboratory features of this case have expanded our knowledge on the ENS neuropathology. First, chronic intestinal partial obstruction occurring as a result of a long-standing mechanical cause (Ladd's band) evokes changes similar to those found in animal models; second, enteric neuropathologic changes observed in the advanced stage of this case could not be differentiated from the neurodegenerative findings often detected in cases of CIPO.

In the current case, early tissue analysis of dilated bowel segments (i.e., from the region which was subsequently identified as proximal to the Ladd's band) showed hyperganglionosis of the ENS. These neuronal changes, which were reported when the patient was 5 years old, may be considered the result of adaptive phenomena to a long-standing mechanical stimulus, such as obstruction limiting bowel propulsion. Several experimental models have been instrumental in supporting the concept that adaptive changes to the ENS occur proximal to a partially obstructed gut[5,15-21]. These studies showed increased density and size in both myenteric[16,18,19,21] and submucosal neurons[19-21], along with neurochemical[18,20] and cytoskeletal abnormalities of myenteric neurons[21]. Recently, Galvez *et al*[20] induced surgical stenosis (about 20% of the lumen) of rat sigmoid colon and reported changes in ENS structure after 6-12 wk. The molecular mechanisms involved in enteric neuroplasticity secondary to a partially obstructed bowel remain to be elucidated. Further, enteric neuronal changes, ICC (the pace-maker cells of the gut, which act in concert with neurons in governing gut motility) have been found to be reduced in experimental mechanical subocclusion of the small bowel as indicated by a decrease in c-Kit immunoreactivity and loss of functions attributed to ICC[17,18]. In contrast, other data do not support the existence of ICC abnormalities (according to c-Kit immunostaining) in dilated gut segments of patients with Crohn's disease, a well-known cause of intestinal mechanical subocclusion[22]. The possible explanations for the discrepancy between this and other studies remain to be defined.

About nine years after the initial observation of hyperganglionosis, the evaluation of new full-thickness biopsies from the dilated bowel above the stenosis showed evidence of a significant loss of both enteric neurons and ICC, which may explain the marked deterioration of the digestive function observed in our patient. Enteric neuron depletion is supported by the evidence of neurodegenerative changes detected by electron microscopy along with the reduced expression of the protein encoded by *BCL-2,*

a gene related to one of the intracellular pathways involved in the inhibition of programmed cell death[2,3,10,11]. These findings indicate that neuronal cell loss may be due to apoptosis triggered by the persistent mechanical sub-occlusion of the gut. The neurodegenerative abnormalities observed in this phase of the clinical history of the patient may resemble ENS changes described both in humans[23] and in an experimental model of small bowel atresia[24]. In human intestinal atresia, Masumoto *et al*[23] have shown hypoplasia of myenteric ganglia and marked reduction of both intramuscular nerve fibers and ICC in the dilated bowel segments above atresia. Similarly, in a chick embryo model of intestinal atresia, Schoenberg and Kluth[24] have found an almost complete loss of both ganglionated plexuses in proximal dilated loops.

Taken together, enteric hyperganglionosis followed by degenerative changes can be considered the result of a bi-phasic adaptive process of the ENS in response to a persistent mechanical obstruction of the gut.

A further consideration which can be drawn from the present case concerns the accuracy of ENS pathology in patients with intestinal sub-occlusion. This case report clearly indicates that the enteric neuropathologic changes observed in different stages of a mechanical obstruction could not be distinguished from the neurodegenerative findings often detected in cases of CIPO. Neuropathology of CIPO includes a wide spectrum of ENS changes ranging from hyperganglionosis up to marked reduction of intramural (especially myenteric) neurons associated with swollen cell bodies, variable neurochemical abnormalities and fragmentation and loss of axons sometimes accompanied with proliferation of glial cells[2-5]. If the mechanical obstruction remains unrecognized, as it did in our case, the ENS abnormalities mimic those identifiable in CIPO associated with an underlying neuropathy. Therefore, our case should be considered as a reminder that ENS changes observed in patients with suspected CIPO may be secondary in nature and should not necessarily be interpreted as definitive evidence for a primary neuropathy.

In conclusion, congenital (partial) mechanical obstruction of the upper small bowel leads to progressive adaptive/neuroplastic changes of the ENS similar to those described in experimental models of intestinal occlusion. Interestingly, such ENS abnormalities mimic those often observed in cases of CIPO.

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CASE REPORT

Unusual gastric and pancreatic metastatic renal cell carcinoma presentation 10 years after surgery and immunotherapy: A case report and a review of literature

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Abstract

Renal cell carcinoma (RCC) is the most common renal tumor, accounting for 2%-3% of all malignancies. Though RCC is known to spread hematogenously, isolated RCC metastasis to the stomach is a rare event. In this article, we describe the clinical course of a patient who developed a pancreatic recurrence of RCC and 1 year later a gastric recurrence of RCC treated 10 years ago with a resection and interleukin-2 (IL-2).

Accumulating evidence indicates that metastatic involvement of the pancreas and stomach should be suspected in any patient with a history of RCC who presents with gastrointestinal symptoms even 10 years after RCC resection and immunotherapy.

Key words: Renal cell carcinoma; Stomach metastasis; Interleukin-2 treatment

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INTRODUCTION

Renal cell carcinoma (RCC) is the most common renal tumor, accounting for 2%-3% of all malignancies[1]. It has the highest incidence in the 6th decade and occurs twice as often in men compared to women[2]. Several risk factors are associated with a high risk for RCC such as tobacco smoking, obesity, exposure to asbestos or chemicals, thiazide drug intake and urinary tract infections[3]. RCC causes only few early warning signs. The symptoms are often non-specific and the triad of Virchow consisting of an abdominal mass together with flank pain, and macroscopic hematuria is nowadays rare[4]. RCC spreads hematogenously and is known for its ubiquitous metastatic patterns. While pancreatic localization of recurrence is quite common[4,5], the gastric localization of recurrences of this type of tumor is quite rare. Some authors have described a recurrence of RCC in the stomach but among them only a few are well documented[6-14].

In this article, we describe a patient who developed a pancreatic recurrence of HCC and 1 year later a gastric recurrence of RCC treated 10 ago before with a resection and IL-2.

To our knowledge, this is the first report that describes the recurrence of metastatic RCC 10 years after a complete response to high-dose IL-2 therapy showing as a pancreatic metastasis and a year later again as a gastric metastasis.

CASE REPORT

This paper reports the history of a 68-year-old male patient. He underwent a right radical nephrectomy for RCC in 1990 followed by a high-dose IL-2 treatment. No other relevant disease was present in his past clinical history.

Ten years after the above-mentioned intervention he developed a disease recurrence involving the spleen and tail of the pancreas. He underwent a standard diagnostic work-up that included hematochemical determinations, then a splenectomy and distal pancreatectomy followed by a high-dose IL-2 therapy. No evidence of involvement of other abdominal organs was present.

One month after the last intervention, he visited the emergency room with a 10-d lasting history of polyuria and polydipsia. His physical examination was negative and hematochemical assays revealed the presence of hyperglycemia (494 mg/dL) and an increase of serum amylase (329 IU/L), while the hematocrit and differential blood counts were normal. The diagnosis was diabetes related to the recent distal pancreasectomy. He was treated with fast-acting insulin and discharged with a

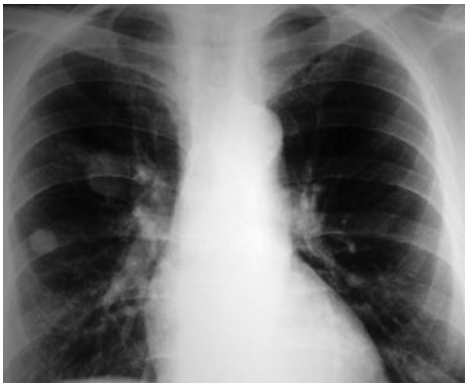

Figure 1 Metastases of 1 and 3.5 cm in diameter as shown by chest X-ray.

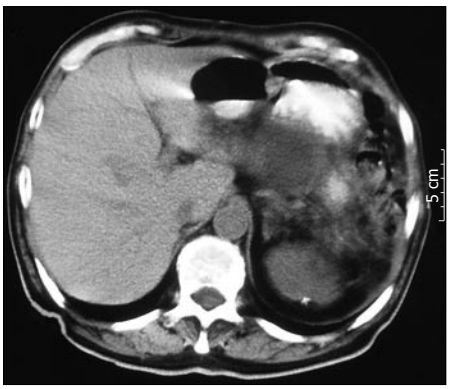

Figure 2 An irregular mass (5 cm in diameter) corresponding to gastric metastasis, several para-aortic lymphoadenopathies and liver metastatic nodules as shown by CT scan of the abdomen.

prescription of insulin. Eleven months later, he again visited the emergency room with a 3-d history of postural dizziness and weakness. He also reported melena the day before admission. The only other gastrointestinal symptom that could be elicited was early increasing satiety after meal during the pervious year. No weight loss was present. Hematochemical determinations revealed anemia (hematocrit 17.7%, hemoglobin 56 g/L) with a rise in the count of platelets (579 000/μL). The level of glycemia at fasting was 453 mg/dL though the patient was following his therapy. No abnormal finding was present on physical examination. A nasogastric tube was placed and fresh blood in the stomach was revealed at aspiration. The patient immediately received intravenous fluids and 3 units of blood. His hyperglycemia was treated with fast-acting insulin. The patient underwent upper gastrointestinal endoscopy showing a multilobulated, polypoid, friable, and bleeding mass (5 cm in diameter) in the gastric fundus with a central bleeding ulcer. Epinephrine was injected into the lesion and the bleeding stopped. The patient underwent a further endoscopy 3 d later and a sample of the mass was taken for biopsy. Histopathologic examination revealed a metastatic RCC which was a poorly differentiated clear cell variant lesion, being consistent with a metastasis of RCC resected 11 years ago. Chest X-ray showed two metastases with a maximum size of 1 and 3.5 cm respectively (Figure 1). Computed tomography (CT) scans of the abdomen displayed a gastric mass (5 cm in diameter), several para-aortic lymphoadenopathies and liver metastases (Figure 2).

Chemotherapy was proposed to reduce the tumor size as well as the hepatic and pulmonary metastases, but the patient refused to undergo any treatment and was discharged.

Table 1 Characteristics of all reported patients with RCC who developed isolated metastatic gastric lesions

Patients (*n*)	Appearance after nephrectomy	Outcome
1	7-years	3-year DFS
1	7-years	Not available
1	4-years	17-mo DFS
1	6-years	> 5-mo survival
6	Not known	Autopsy study
2	Not known	Autopsy study
1	9-years	Not available
1	Not known	Autopsy study
1	At nephrectomy	3-mo survival
1	13-years	2-year survival

RCC: Renal cell carcinoma; DFS: Disease-free survival.

After one month, he presented again to the emergency room with postural dizziness, melena, and weakness. The patient eventually underwent surgery. The large mass described above was found in the posterior wall of the fundus and body of the stomach. It was penetrating the surrounding tissues. Many lymphoadenopathies along the common hepatic artery, left gastric artery and celiac trunk were also present. Therefore, total gastrectomy was performed with lymphoadenectomy and omentectomy, and esophago-jejunostomy according to the Roux technique. Histopathological examination confirmed the presence of metastatic clear RCC. Both proximal and distal resection margins appeared to be tumor free. The patient had an uneventful postoperative course.

One month after the intervention, he was started on chemotherapy with gemcitabine and IL-2. The therapy lasted for 20 mo. The patient during this period had a good quality of life. Because of the presence of abdominal pain, a treatment with morphine was started. He also received erythropoietin and iron to treat anemia. Then he received blood transfusions in the following 3 mo. The patient finally died due to the worsening of anemia and cachexia.

DISCUSSION

This paper describes for the first time an uncommon biological and clinical behavior of RCC. RCC is known to cause metastatic recurrences many years after its resection and even after immunotherapy[14]. However, while recurrence at the pancreatic site is quite common, gastric recurrence is atypical[15]. Metastatic involvement of the stomach is usually considered as an extraordinary event and accounts for only 0.2%-0.7% of gastric neoplastic diseases. In a large series consisting of 23 019 autopsies, Davis and Zollinger[16] found 67 metastases to the stomach from primary tumors outside the gastrointestinal tract. None was from the kidney. Higgins[17] reported 64 metastases in the stomach out of 31 541 examined autopsies. In this series, none was from the kidney.

An isolated gastric metastasis from RCC is extremely rare and only 15 cases including the present one have been described[6-14]. The characteristics of all the reported cases

including our one are summarized in Table 1. From Table 1, we could see that RCC metastasizes to the stomach several years after nephrectomy.

Gastric RCC metastasis often starts as a submucosal lesion, which encroaches onto the mucosa and becomes ulcerated. The lesion may be single or multiple and is grossly polypoid or plaque-like. Metastatic tumors can be distinguished from gastric carcinoma based on the absence of cellular atypia in the gastric glandular structures which may appear compressed by the metastatic tumor. The most common presenting symptom is abdominal pain but nausea, vomiting, and gastrointestinal bleeding can also be present. Gastrointestinal bleeding is mainly related to acid erosion of the metastatic lesion. Diagnosis is confirmed by upper gastrointestinal endoscopy and histopathological examination of a biopsy sample of the lesion.

Surgical excision of gastric metastasis is mandatory as these lesions may bleed again after endoscopic coagulation treatment. A unique metastasis should be treated as a new tumor with prompt surgical excision. This often results in a significant survival prolongation with a good quality of life. In the present case, though multiple pulmonary and hepatic metastases were present, surgery had to be performed because of the high risk of rebleeding.

Compared to previously reported cases, our patient had the longest disease-free interval from the resection of RCC to the stomach metastasis as he developed recurrence at the pancreatic site 10 years after the resection of RCC and gastric metastasis 1 year later.

The prolonged disease-free time could be at least in part due to the IL-2 immunotherapy that apparently yielded a complete response. Immunotherapy with IL-2 is in fact considered as the mainstay of treatment for RCC as cytotoxic chemotherapy fails[18]. This treatment can achieve certain effects in patients with a metastatic disease[19]. The mechanism of action of IL-2 at the cellular level is not perfectly known. It is hypothesized that IL-2 stimulates a cellular immune response to tumor involving mainly the natural killer cells and T lymphocytes[20]. The response rate fluctuates from 15% to 20% and complete responders account for one-third of this group[19]. The survival time of complete responders is significantly prolonged and exceeds 60% at 5 years, while non-responders with metastatic RCC have a median survival time of less than 1 year[19].

The present case draws attention to an important aspect of the biological behavior of RCC. This tumor is often a slow-growing lesion and may be associated with a late onset of solitary metastases that may occur even 11 years later than the primary tumor. Usually metastases grow slowly. In our patient, the first pancreatic recurrence was 1 year earlier than the gastric one. Gastric localization was undetectable when the patient underwent surgery for the first recurrence. Therefore, a careful follow-up should be made in patients who develop a first recurrence of RCC since they may develop multiple recurrences at different sites even after several years. IL-2 therapy seems to be able to treat the disease once it spreads out but has no effect on its prevention.

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CASE REPORT

Pharmacokinetics of paclitaxel in a hemodialysis patient with advanced gastric cancer: A case report

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Abstract

We report for the first time the possibility of weekly paclitaxel chemotherapy for a patient with advanced, nonresectable gastric cancer undergoing hemodialysis. A 50-year-old man with chronic renal failure due to bilateral polycystic kidneys, who had undergone hemodialysis three times a week for 5 years, presented with hematemesis in December 2004. Based on the diagnosis of gastric cancer with lymph node metastases, surgery was performed. On the 15th postoperative day, the patient was treated with chemotherapy using paclitaxel. Paclitaxel was administered at a dose of 60 mg/m^2 as a 1 h iv infusion in 250 mL of saline. Hemodialysis was started 1 h after the completion of the paclitaxel infusion and was performed for 3 h. Paclitaxel was administered weekly on d 1, 8, and 15 on a 28-d cycle. The maximum plasma concentration of paclitaxel was 1390 μg/L. The area under the curve of paclitaxel was 4398.6 μg·h/L. Grade 2 leukopenia was encountered during the first cycle. The plasma concentrations of paclitaxel from 6 to over 24 h after the infusion were 0.01 to 0.1 μmol/L in our patient, and these concentrations have been shown to be effective on inhibiting the growth of gastric cancer cells without producing adverse side effects in the patient. The plasma concentration of paclitaxel was not influenced by hemodialysis. We conclude that the pharmacokinetics of paclitaxel is not altered in a patient with renal failure, and that weekly paclitaxel is a suitable treatment regimen for hemodialysis patients with advanced gastric cancer.

Key words: Paclitaxel; Gastric cancer; Hemodialysis

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INTRODUCTION

The necessity of chemotherapy for hemodialysis patients with malignancies has increased as the life span of hemodialysis patients has grown longer because of improvements in hemodialysis. The median survival time of patients receiving the best supportive care for advanced, nonresectable gastric cancer (AGC) is only 3 to 4 mo. Some randomized studies have demonstrated the superiority of chemotherapy over best supportive care for improving patient prognosis, and the significance of performing chemotherapy for patients with AGC is now recognized[1,2]. Although TS-1 has frequently been used for patients with AGC as a first line chemotherapy in Japan, this drug is prohibited for use in patients with renal failure because adverse reactions, such as bone marrow depression, may be enhanced. In this case report, we describe for the first time the possibility of weekly paclitaxel chemotherapy for a hemodialysis patient with AGC.

CASE REPORT

A 50-year-old man with chronic renal failure due to bilateral polycystic kidneys, who had undergone hemodialysis 3 times a wk for 5 years, presented with hematemesis in December 2004. Endoscopic examination demonstrated a large tumor on the lesser curvature of the antrum. Biopsy specimens of the tumor revealed poorly differentiated adenocarcinoma. Computed tomography of the abdomen showed enlarged lymph nodes surrounding the stomach and displayed bilateral polycystic kidneys. Based on the diagnosis of gastric cancer with lymph node metastases, surgery was performed. On entering the peritoneal cavity, peritoneal disseminations were encountered. Although a curative intervention was not possible, distal gastrectomy was performed to prevent bleeding from the cancerous lesion. On the 15th postoperative d, the patient was treated with chemotherapy using paclitaxel.

Paclitaxel was administered at a dose of 60 mg/m^2 as a 1 h iv infusion in 250 mL of saline. The following premedication was administered as a 60 min pretreatment prior to paclitaxel: dexamethasone, 10 mg iv; chlorphenamine, 10 mg iv; and ranitidine, 50 mg iv. Hemodialysis was started 1 h after the completion of the paclitaxel infusion and was performed for 3 h. Paclitaxel was administered weekly on

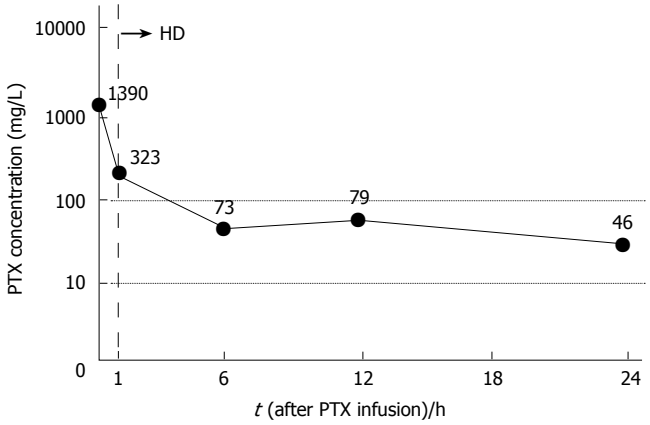

Figure 1 The plasma concentrations of paclitaxel following iv infusion. PTX: Paclitaxel; HD: Hemodialysis.

d 1, 8, and 15 on a 28-d cycle.

The plasma concentrations of paclitaxel following iv infusion are shown in Figure 1. The maximum plasma concentration (Cmax) of paclitaxel was 1390 μg/L. The area under the curve (AUC) of paclitaxel was 4398.6 μg·h/L. Grade 2 leukopenia occurred during the first cycle. Chemotherapy had to be stopped because bleeding from the jejunum near the anastomosis occurred, in spite of the fact that the platelet count was normal during the first cycle. Unfortunately, peritonitis carcinomatosa occurred, and the patient died in April 2005.

DISCUSSION

Anticancer drugs such as 5-FU, cisplatin, and irinotecan hydrochloride are effective for gastric cancer, but in a patient with hemodialysis-dependent renal failure, the dosages of these drugs must be carefully chosen because of potential side effects arising from the increased blood concentration of these drugs. Paclitaxel is the first drug from a group of drugs that inhibit microtubule disaggregation and is extensively metabolized by the liver and secreted in bile, with less than 10% extracted by the kidneys[3,4]. As a single agent, phase II study results on gastric cancer have demonstrated an overall response rate of approximately 15% to 20%[5,6]. Paclitaxel has a cytotoxic effect on human gastric cancer cell lines in a dose- and time-dependent manner. Chang *et al*[7] reported that exposure of gastric cancer cells to 0.01 μmol/L of paclitaxel for 24 h appeared to be cytotoxic. On the other hand, some investigators have reported that a paclitaxel threshold of 0.1 μmol/L was informative with respect to neutropenia, and that the total dose and AUC did not correspond with the incidence or severity of neutropenia[8,9]. In our phase I study of weekly paclitaxel and doxifluridine in AGC patients, the recommended dose of paclitaxel was 80 mg/m^2 plus doxifluridine at 533 mg/m^2. However, the effective rate was 33.3% with no adverse events at a paclitaxel dose of 60 mg/m^2[10,11]. Therefore, we decided to use a dose of 60 mg/m^2 of paclitaxel for the first cycle to ensure the safety of our patient. To our knowledge, there are a small number of reports in the literature on the treatment of advanced ovarian cancer with paclitaxel in patients with renal failure, but there are no reports of chemotherapy with paclitaxel in an AGC patient with renal failure[12,13]. The plasma concentrations of paclitaxel at 6 to over 24 h after the infusion were 0.01 to 0.1 μmol/L in our patient, and these concentrations have been shown to be effective on inhibiting the growth of gastric cancer cells without producing adverse effects for patients. Kim *et al*[14] reported the pharmacokinetic analysis in the treatment of relapsed breast cancer by weekly paclitaxel. In that report, the peak concentrations at 0 min (Cmax), 30 min, and 24 h in patients treated with a dose of 60 mg/m^2 of paclitaxel were 2.18 ± 0.68, 0.65 ± 0.20, and 0.017 ± 0.012 μmol/L, respectively. These findings are similar to our results and suggest that the plasma concentration of paclitaxel is not influenced by hemodialysis.

Chemotherapy has to be stopped because of bleeding from the postoperative peptic ulcer. Neither thrombocytopenia nor coagulopathy occurs as a result of chemotherapy. We conclude that the pharmacokinetics of paclitaxel is not altered in a patient with renal failure and that weekly paclitaxel is a suitable treatment regimen for patients with AGC on hemodialysis.

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CASE REPORT

Development of ulcerative colitis during the course of rheumatoid arthritis: Association with selective IgA deficiency

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Abstract

A 56-year-old woman with a 29-year history of rheumatoid arthritis (RA) was admitted to the hospital, complaining of high fever, abdominal pain and severe bloody diarrhea. Colonoscopy revealed friable and edematous mucosa with spontaneous bleeding, diffuse erosions and ulcers extending from the rectum to the distal transverse colon. Histopathological findings of rectal biopsies were compatible with ulcerative colitis (UC). Being diagnosed as having severe active left-side UC, she was successfully treated with intravenous methylprednisolone followed by prednisolone and leukocytapheresis. Laboratory tests revealed low serum and saliva IgA levels, which might play a role in the development of UC. To our knowledge, this is the first case of UC occurring during the course of RA, accompanied by selective IgA deficiency.

Key words: Ulcerative colitis; Rheumatoid arthritis; Selective IgA deficiency

Asada Y, Isomoto H, Shikuwa S, Wen CY, Fukuda E, Miyazato M, Okamoto K, Nakamura T, Nishiyama H, Mizuta Y, Migita K, Ito M, Kohno S. Development of ulcerative colitis during the course of rheumatoid arthritis: Association with selective IgA deficiency. *World J Gastroenterol* 2006; 12(32): 5240-5243

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INTRODUCTION

Recent case reports described the development of ulcerative colitis (UC) in patients with rheumatoid arthritis (RA)[1,2]. Although UC is commonly associated with arthritic manifestations such as ankylosing spondylitis and sacroilitis[3], RA complicated by UC is uncommon[4], hence the underlying mechanisms of the association between UC and RA remain unclear. Differentiation between UC and colitis, primarily or secondarily related to RA or drug-induced colitis associated with medications for RA, can be difficult sometimes[5].

Selective deficiency of immunoglobulin A (IgA) is the most frequent hypogammaglobulinemia[6]. It is associated with increased risk of autoimmune diseases including RA[6,7]. One might speculate that IgA deficiency plays a role in the mucosal immune and inflammatory responses of UC[6]. Herein, we describe the development of UC in a patient with longstanding RA and selective IgA deficiency. To the best of our knowledge, this is the first case report on the association of RA with UC and IgA deficiency.

CASE REPORT

A 56-year-old woman who had been diagnosed with UC at a local clinic 5 mo earlier, was admitted to our hospital, complaining of high fever, exacerbated lower abdominal pain and a 4-wk duration of bloody diarrhea, despite treatment with 30 mg/d of oral prednisolone. She had suffered from RA for 29 years and the current stage and class of RA, based on Steinbrocker's classification, was Ⅳ and Ⅱ, respectively. RA had been stable for the last 5 years under continuous treatment consisting of bucillamine (100 mg/d), prednisolone (5 mg/d) and D-penicillamine (600 mg/d). She had a history of adverse events of proteinuria and skin eruption with itching against bucillamine and mesalazine, respectively. She had not taken any other medications such as antibiotics, non-steroid anti-inflammatory drugs (NSAIDs) or gold salts, prior to the development of UC. She was a non-smoker, did not drink alcohol and had not undergone pervious gastrointestinal surgery.

On admission, physical examination revealed body weight of 32 kg, height of 146 cm, body temperature of 37.9℃, regular pulse rate of 96/min and blood pressure of 148/76 mmHg. Conjunctiva palpebra and bulbus were

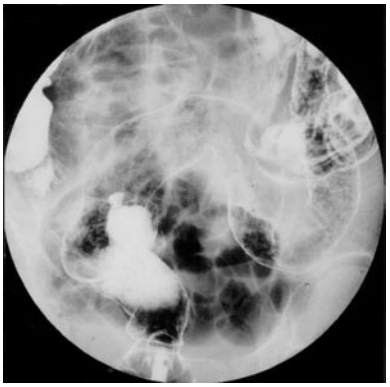

Figure 1 Barium enema shows loss of mucosal pattern with speculation, absent haustration, narrowing and shortening of the bowel, extending from the rectum to the distal transverse colon.

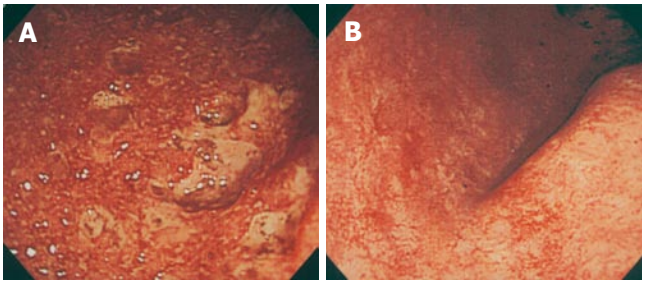

Figure 2 (**A**) Sigmoidoscopy revealed friable, edematous mucosa with granularity, mucous exudates and bleeding throughout the rectum and sigmoid colon. Multiple erosions and punctate ulcers were observed without normal-appearing intervening mucosa. (**B**) Repeated colonoscopy before discharge revealed almost normal mucosa from the distal transverse colon to the rectum.

normal without pallor or jaundice. Heart sounds were clear with regular sinus rhythm and no rales were audible in the lung fields. The abdomen was tender on palpation in the lower quadrant with weak bowel sounds, but neither muscular guarding nor Blumberg's sign was noted. Digital rectal examination revealed dark red blood. There was no edema on the face or extremities and there were no signs of motor or sensory disturbances. Typical joint deformities and morning stiffness of RA were noted in both hands.

Laboratory investigations showed leukocyte count, 10900/mm^3 (normal, 3500-9100); platelet, 30.6 × 10^4/mm^3 (normal, 13.0-36.9 × 10^4); hemoglobin (Hb), 132 g/L (normal, 113-152); C-reactive protein (CRP), 6.6 g/L (normal, < 0.3); serum total protein, 53 g/L (normal, 65-82); albumin, 23 g/L (normal, 37-52); IgG, 16.58 g/L (normal, 8.7-17); IgA, 0.2 g/L (normal, 1.1-4.1); IgM, 0.63 g/L (normal, 0.46-2.6); rheumatoid factor, 48000 IU/L (normal, 0-20000); passive agglutination (RAPA), 1:80 (normal, < 1:40); antinuclear antibodies < 40 (normal, < 40); CH50, 23.2 g/L, (normal, 25-48); and C3, 0.63 g/L (normal, 0.86-1.6). Saliva IgA level was low at 37.6 mg/L (normal, 51.6-931). Urinalysis was normal and no infectious agents were identified in stool specimens. *Clostridium difficile* toxin was negative in feces.

Chest X-ray revealed no abnormalities such as pneumonia and interstitial pneumonitis. Abdominal plain X-ray revealed a small amount of bowel gas without formation of air-fluid level or free air and no toxic dilation of the colon was seen. Hand X-rays revealed bilateral involvement of most metacarpal phalangeal joints with destruction and deviation, whereas systemic skeletal X-ray

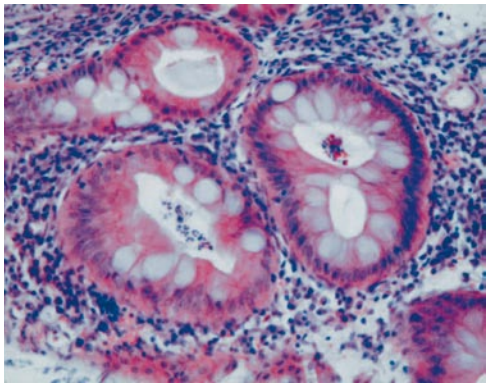

Figure 3 Histopathological examination of biopsied rectal mucosa showed severe neutrophil infiltration, goblet cell depletion and mild crypt abscess (hematoxylin and eosin staining).

study showed no abnormalities in cervical and lumbar spine and hip joints. Barium enema showed disappearance of haustra coli, narrowing and shortening of the affected colon, and uneven surface with spiculation, extending from the rectum to the distal transverse colon (Figure 1). The terminal ileum and the ascending and proximal transverse colon were intact. Barium study of the small intestine and upper gastrointestinal endoscopy were normal. Contrast computed tomography of the abdomen and pelvis showed diffuse mural thickening of the affected colon. Initial sigmoidoscopy performed on the next hospitalization day revealed friable, edematous mucosa with granularity, mucous exudates and spontaneous bleeding throughout the rectum and sigmoid colon. Diffuse erosions and punctate ulcers were noted with lack of normal-appearing intervening mucosa (Figure 2A). Histopathology of colorectal biopsies showed diffuse mucosal infiltration of inflammatory cells, deformed atrophic crypts, goblet cell depletion and crypt abscess (Figure 3). No granulomas, inclusion bodies, vasculitic lesions or amyloid deposits were detected under microscopic examination of hematoxylin-eosin and Congo-red stained sections (data not shown). Based on the clinical, radiological, endoscopic, and histopathological findings, the patient was diagnosed as having severe active, left-sided UC.

Under total parenteral nutrition, she received a course of intravenous methylprednisolone pulse therapy (1 g/d for 3 consecutive days), followed by large doses of intravenous prednisolone, starting with 50 mg/d. Since cytomegalovirus antigenemia was detected 7 d after admission, she also received a 14-d course of intravenous gancyclovir injection at 400 mg/d.

She also received leukocytapheresis (LCAP) therapy, employing a commercially available leukocyte removal column (Cellsorba, Asahi Medical, Tokyo) once a week for 10 successive weeks. Such intensive treatment led to dramatic improvement of clinical symptoms and signs without any adverse reaction and to normalization of laboratory tests. Follow-up colonoscopy 3 wk later revealed marked improvement, and normal colonic mucosa at discharge from the hospital three months after admission (Figure 2B). The RA disease status was unchanged during hospitalization and thereafter. At the last visit to the outpatient clinic, 6 mo after discharge, the patient was still

in complete remission of colitis under 10 mg/d of oral prednisolone.

DISCUSSION

When colitis appears in RA patients, drug-induced colitis, ischemic colitis due to vasculitis associated with RA and secondary amyloidosis must be ruled out first [8-12]. It is known that therapeutic agents for RA such as NSAIDs, gold salts and D-penicillamine give rise to various types of gastrointestinal complications, evidences indicate that colitic lesions are associated with these anti-RA drugs[8-10]. Although Langer *et al*[9] reported a case of gold salts-induced colitis resembling UC, our patient was not treated with gold salts or NSAIDs. There are also reports on D-penicillamine-induced colitis[10], with rapid recovery after cessation of the agent. Although the patient had used penicillamine for 6 years, colitis had never appeared. Moreover, her colitis improved despite continuous use of this drug. Thus, anti-RA medications are unlikely to be the underlying cause of colitis in our patient.

Rheumatoid vasculitis (also known as malignant RA) affects a variety of organs including kidneys, lungs and gastrointestinal tract[13]. Previous studies indicate that clinically apparent rheumatoid vasculitis occurs in less than 1% of RA patients and intestinal involvement is noted in about 20% of such cases[11]. The gastrointestinal lesions associated with rheumatoid vasculitis are characterized by multiple sharply-defined ulcers, typically spared by normal-appearing mucosa, with a predilection for the small intestine, the sigmoid colon and cecum[8,11]. Burt *et al*[11] described a patient with RA who had colonic vasculitis presenting as pancolitis similar to UC. In that patient, however, repeated colonoscopy following treatment with prednisolone showed that the intervening mucosa among superficial ulcers was normal, allowing differentiation of colitis due to RA-related vasculitis from idiopathic UC. Histopathological examination of multiple biopsies taken from colorectal mucosa identified no vasculitic lesions in our patient, who showed no other vasculitic diseases such as scleritis and interstitial pneumonitis[13,14].

Recent clinical studies have indicated that the incidence of amyloidosis confirmed by biopsies in RA patients is about 10%[12]. Kato *et al*[15] reported a suggestive case of gastrointestinal amyloidosis secondary to RA manifested as pancolitis resembling UC. In our patient, however, rectal biopsy specimens showed diffuse infiltration of mono- and poly-nuclear leukocytes and crypt abscess, which were compatible with UC, but no intramucosal amyloid deposits. In addition, neither microbiological nor histopathological examinations showed any pathognomonic findings in this case, thus allowing differentiation between UC and other possible inflammatory bowel conditions.

Being diagnosed as having severe active UC extending to the left-side colon, the patient was treated with intravenous methylprednisolone and subsequently with prednisolone. Given that the patient had already received oral prednisolone even at 30 mg/d with exacerbation of UC, she was also treated with LCAP. In previous studies, LCAP frequently led to significant improvement of UC that had been intractable to steroids[16,17]. A recent open-label multicenter study demonstrated that LCAP can be more effective than high-dose steroid treatment with fewer adverse effects[17]. Currently, LCAP is used as a treatment modality for active UC. Although histopathological examination of colorectal biopsies showed no inclusion bodies, cytomegalovirus antigenemia was detected, allowing our patient to undergo additional treatment with intravenous gancyclovir. We believe that the intensive combination therapies led to complete clinical and endoscopic remission of severe and steroid-refractory colitis.

Arthritic manifestations represent the most common extraintestinal manifestations of inflammatory bowel diseases, affecting 31% and 36% of patients with UC and Crohn's disease, respectively[5]. Coexistence of RA and UC is, however, rare and no more than 0.14% to 0.8% of UC patients suffer from RA[4,18]. Larger population-based studies are warranted to unravel the mechanisms underlying the association between UC and RA. In this regard, accumulation of HLA data may provide a potential clue for this relationship[19].

In the present case, serum concentrations of IgA, but not IgG or IgM, were constantly low ranging from 1 to 20 mg/dL and reduction of secretory IgA in saliva was also noted during hospitalization. Systemic and local reduction of IgA might result in weakening of the mucous barrier against intestinal flora and enhance exposure of the mucosal immune system to intestinal bacteria or the toxic components, eventually leading to sustained inflammation within the colorectal mucosa[4]. However, the precritical serum IgA level had not been estimated in our patient, and the etiology of selective IgA deficiency remains elusive as to whether it occurred primarily or not. In fact, secondary IgA deficiency may be caused by such anti-RA drugs as D-penicillamine, steroids, both of which had been prescribed for her RA, sulfasalazine and gold salts[7,20].

Liblau *et al*[6] reported that the frequency of autoimmune diseases in IgA deficient patients ranged from 7 to 36%, supporting the notion that selective IgA deficiency may be a risk factor of organ-specific and systemic autoimmune diseases. A recent study showed that the prevalence of IgA deficiency in RA patients was quite low (0.3%)[6], whereas Badcock *et al*[7] reported that IgA deficient patients were more likely to have a history of RA in the first-degree relatives, suggesting inheritance of predisposing factor(s). Of note, Snook *et al*[4] reported that 6.6% of UC patients had at least one autoimmune disorder and the prevalence of a specified group of autoimmune disorders was three times greater than expected in patients with UC.

In conclusion, we described the first case of RA, complicated with UC and selective IgA deficiency. Underlying immune dysfunction, possibly in addition to genetic predisposition, might play a role in these associations.

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CASE REPORT

Prolonged cholestasis after raloxifene and fenofibrate interaction: A case report

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Abstract

Assigning causality in drug-induced liver injury is challenging particularly when more than one drug could be responsible. We report a woman on long-term therapy with raloxifen who developed acute cholestasis shortly after starting fenofibrate. The picture evolved into chronic cholestasis. We hypothesized that an interaction at the metabolic level could have triggered the presentation of hepatotoxicity after a very short time of exposure to fenofibrate in this patient. The findings of an overexpression of vascular endothelial growth factor in the liver biopsy suggest that angiogenesis might play a role in the persistance of toxic cholestasis.

Key words: Raloxifene; Fenofibrate; Drug-drug interactions; Hepatotoxicity; Causality assessment

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INTRODUCTION

Fibrates are extensively prescribed for patients with primary hypertriglyceridemia. Raloxifene, a selective estrogen receptor modulator that prevents bone resorption without causing endometrial or breast cancer, is effective in the treatment of osteoporosis in postmenopausal women. Both agents are widely used in clinical practice, thus posing an increased likelihood of their concomitant prescription. Although hepatotoxicity is a rare phenomenon associated with their use[1-3], we report a patient on long term therapy with raloxifene who developed prolonged cholestatic hepatitis shortly after the introduction of fenofibrate.

CASE REPORT

A 60-year-old woman was started on raloxifene hydrochloride (60 mg/d) in 2000 for the prevention of osteoporosis. On December 1st 2003 she was prescribed fenofibrate (250 mg/d) for hypertriglyceridemia (triglyceride level 423 mg/dL). Her liver function was normal and she had no toxic habits, no drug allergies and was not taking other drugs. There was no family history of cholestatic and non-cholestatic diseases of the liver and biliary tract. She did not suffer from diabetes mellitus or pancreatitis and was moderately obese (BMI 26.7). On December 14th she noticed dark urine and both drugs were discontinued, no attempt of drug reintroduction was recorded. On admission (December 16), she was afebrile and jaundiced. Aspartate aminotransferase (AST) was 153 U/L (normal < 30), alanine aminotransferase (ALT) 241 U/L (normal < 36), alkaline phosphatase (AP) 174 U/L (normal < 104), gammaglutamyltransferase (GGT) 271 U/L (normal < 32), and total bilirubin 11.07 mg/dL with direct bilirubin 9.6 mg/dL. The leucocyte count was 3.3×10^9/L with 17.7% lymphocytes. Serology ruled out viral causes and screening for autoantibodies was negative. Imaging testing including a magnetic resonance cholangiography showed cholelithiasis with no other pathological findings (i.e. gallstones, tumour). A liver biopsy showed a moderate inflammatory infiltrate of lymphocytes with hepatocellular cholestasis and focal necrosis. Immunohistochemistry showed that expression of vascular endothelial growth factor (VEGF) (clone C-1, Santa Cruz Biotechnology, IC, USA) resulted in mild to moderate granular staining in hepatocytes of zone 3 (Figure 1A). Immunohistochemical staining was performed as previously described[4]. Ten days later a morbiliform and very pruriginous rash appeared in the lower extremities that progressively generalized to the trunk, upper extremities and face.

Liver enzymes decreased initially but on January 20, 2004, the total bilirubin peaked at 21.84 mg/dL (direct

Table 1 Liver tests while on raloxifene therapy and follow-up after raloxifene and fenofibrate interaction during 14 d of coadministration

Admission date	Nov 10, 2003	Dec 16, 2003	Dec 22, 2003	Jan 2, 2004	Jan 20, 2004	Mar 1, 2004	April 16, 2004	July 2, 2004	Aug 13, 2004	February, 2005	August, 2005
Serum Bilirubin (mg/dL)											
Total	-	11.07	14.91	14.33	21.84	8.31	2.81	0.72	0.78	0.65	0.83
Direct	-	9.58	14.07	13.69	19.72	7.72	1.79	0.14	-	-	-
AST (U/L)	26	153	88	36	321	97	106	84	84	45	53
ALT (U/L)	16	241	167	50	382	160	162	91	74	45	60
GGT (U/L)	21	271	239	97	1246	1387	539	1279	1255	751	666
Alkaline phosphatase (U/L)	72	174	236	240	800	420	490	1195	793	493	444
Lymphocytes (%)	26	17.7	13.8	11.5	5.9	14.3	17.7	-	27.7	33.9	32.20
VEGF (pg/mL)	-	-	-	-	144	-	-	244	-	-	-

Treatment was stopped on 14 December 2003. Total bilirubin (n < 1.8 mg/dL); AST: Aspartate aminotrasferase (n < 30 U/L); ALT: Alanine aminotransferase (n < 36 U/L); GGT: Gamma-glutamyl transpeptidase (n < 32 U/L); Alkaline phosphatase (n < 104 U/L); lymphocytes (n = 20%-40%); VEGF: Plasma vascular endothelial growth factor.

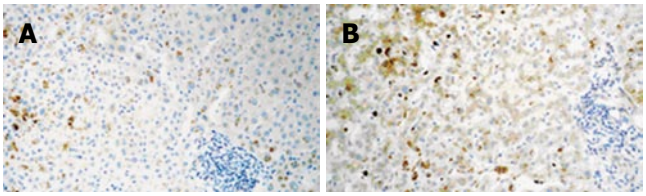

Figure 1 **A**: The first liver biopsy in December 2003 showing mild to moderate granular stain in hepatocytes of zone 3; **B**: The second liver biopsy in February 2004 showing diffuse VEGF stain with predominance of zone 3 (original magnification 200 x).

bilirubin 19.7 mg/dL), with increases in ALT, GGT and AP values (Table 1). Lymphocytes were 0.077 × 10^9 /L (5.9%). The multiformed erythema worsened at this time with subsequent resolution. In February, a second liver biopsy was performed (Figure 1B). The liver specimen showed intense hepatocellular and canalicular cholestasis, mainly in zone 3, with mild to moderate lymphocytic infiltrate in portal tracts (one of them with focal interface hepatitis). Biliary ducts were absent in three out of the 7 portal tracts present in the biopsy. In this biopsy VEGF showed diffuse positivity which was more intense in zone 3, and Kupffer cells were also reactive. At this time, EDTA plasma VEGF levels (determined using a commercially available ELISA kit, R&D Systems, MN, USA) were 144 pg/mL (healthy subjects levels < 80 pg/mL)[5]. On July 1, 2004, ALT was 91 U/L, AP 1195 U/L, GGT 1279 U/L, and total bilirubin 0.72 mg/dL. Plasma VEGF values increased to 244 pg/mL. ALT, GGT and AP levels still remained high 21 mo after the initial episode (Table 1).

DISCUSSION

Assigning a causal relationship to a drug associated with hepatic injury remains a major challenge, especially when it is the first report of a particular reaction and when more than one drug could be the culprit. If treatments are not started simultaneously, common sense and causality assessment methods tend to incriminate the last drug introduced, as the fenofibrate in our patient. Actually, fenofibrate scored higher than raloxifene when the CIOMS scale was applied to yield 9 and 6 points which fell in the category of highly probable and probable, respectively.

However, this issue may not always be so straightforward and other considerations should be born in mind. Acute hepatitis is rarely related to fenofibrate and the reported cases do not reflect the type of injury that is presented here. Most cases present with hypergammaglobulinemia and high titers of anti-nuclear antibodies, and on liver biopsies a lympho-plasmocytic infiltrate, resembling type I auto-immune hepatitis, is evident[1]. The chronic forms of liver damage are more exceptional, usually appearing after long periods of exposure. They show different histopathological findings such as chronic active hepatitis with bridging necrosis or a reduction in the number of interlobular bile ducts in a clinically asymptomatic patient[2]. On the contrary, the only published case of raloxifene-associated hepatitis did exhibit a late peak of bilirubin one month after drug withdrawal [3], similarly to our patient.

To our understanding, this patient suffered from hepatic toxicity due to an interaction between raloxifene and fenofibrate that could result in liver toxicity by altering the threshold for exposure to toxic metabolites. Both compounds are highly protein bound to albumin with the potential of competitive drug displacement[7], and an irreversible inhibition of CYP3A4 by raloxifene has been described which is more frequently associated with unfavorable drug-drug interactions[8,9].

The prolonged course of the abnormalities in liver biochemistry deserves further consideration. Indeed, an immune mechanism is suggested in the patient by the presence of a severe toxic cutaneous reaction and cytopenia. In these circumstances, a self propagating immune response may persist, which might explain the outcome[6].

An interesting finding in this case was the overexpression of VEGF (the most potent proangiogenic growth factor) in the liver and increased plasma VEGF levels, when the clinical and biological picture was in remission and the hepatic lesion evolved into a chronic

cholestatic phase. This suggests that angiogenesis may be an important mechanism involved in the persistence of toxic cholestasis which is up-regulated in response to tissue damage and release of pro-inflammatory cytokines. This has been recently shown in primary biliary cirrhosis[10]. Indeed, angiogenesis is a novel mechanism involved in chronic liver damage and its role in drug-induced liver injury also deserves to be defined.

In summary, an interaction between raloxifene and fenofibrate may occur in a postmenopausal woman with resulting hepatotoxicity. Clinicians should be aware of this adverse reaction and patients should be followed up closely. Clinical judgment of the attribution of causality must be made, especially in particularly troublesome cases in which major drug metabolism mechanisms and the potential for pharmacokinetic drug interactions should always be kept in mind.

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CASE REPORT

Spinal cord compression secondary to bone metastases from hepatocellular carcinoma

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Abstract

Bone metastases are rare in primary hepatocellular carcinoma (HCC). Spinal cord compression (SCC) due to bone metastases occur commonly in patients with lung and breast carcinomas, and metastatic HCC is an unusual cause of SCC. Spinal cord compression is an oncologic emergency and treatment delays can lead to irreversible consequences. Thus, the awareness that SCC could be a potential complication of bone metastases due to HCC is of significance in initiation of early treatment that can improve the quality of life and survival of the patients, if diagnosed earlier. This paper describes four cases of primary HCC with varied manifestations of SCC due to bone metastases. The first patient presented primarily with the symptoms of bone pains corresponding to the bone metastases sites rather than symptoms of associated hepatic pathology and eventually developed SCC. The second patient, diagnosed as having HCC, developed extradural SCC leading to paraplegia during the course of illness, for which he underwent emergency laminectomy with posterior fixation. The third patient developed SCC soon after the primary diagnosis and had to undergo emergency laminectomy. Post laminectomy he had good neurological recovery. The Fourth patient presented primarily with radicular pains rather than frank paraplegia as the first manifestation of SCC.

Key words: Alpha feto protein; External beam radiotherapy; Hepatocellular carcinoma; Laminectomy; Spinal cord compression

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary tumor of the liver and has a mean survival of less than one year after primary diagnosis, if left untreated[1]. Extra-hepatic spread of HCC occurs commonly to the lungs (37%-70%) and regional lymph nodes (23%-45%)[1]. The reported incidence of bone metastases is comparatively low (2%-20%)[2-5]. However, an incidence of 28% of bone metastases from HCC has been reported by Katyal *et al*[1]. The most frequent sites of bone metastases reported by Kuhlman *et al* were ribs, spine, femur, pelvis and humerus[2]. Spinal cord compression (SCC) is a rare event in the natural course of metastatic HCC as evidenced by Bharat Kantharia *et al* in their study on HCC[6]. Review of literature shows only a few patients with HCC who developed radiculopathy or SCC secondary to bone metastases. SCC is an oncologic emergency and awareness that SCC could be a potential complication of bone metastases, can improve the survival and quality of life of these patients, if treated promptly. We herein report four cases of pathologically confirmed primary HCC with spinal metastases who developed extradural SCC.

CASE REPORTS

Case 1

A 55-year-old normotensive, nondiabetic, non-alcoholic man with no history of chronic liver disease, presented with the complaints of low backache and generalized weakness for 20 d. Clinically, he had tenderness in the presacral region. Further investigations revealed normal hematology and biochemical parameters. Magnetic resonance imaging (MRI) of the whole spine showed a tumor deposit in the presacral area. Whole body 99 mTc bone scan revealed increased radiotracer uptake in right ilium, sacrum including both sacroiliac joints and D3 vertebra. Computerised Tomography (CT) guided biopsy and fine needle aspiration cytology (FNAC) from the presacral tumor were consistent with the metastatic

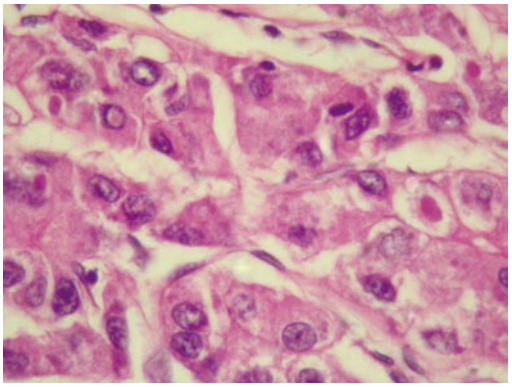

Figure 1 Tru cut biopsy of liver lesion showing increased trabecular thickness, cellular pleomorphism with vesicular nuclei. Sinusoids are lined by flat endothelial cells, Kupffer cells are absent.

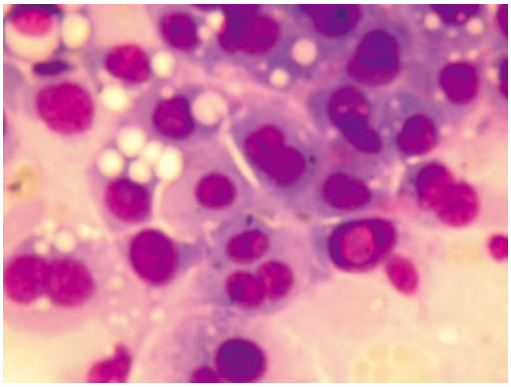

Figure 3 FNAC of vertebral metastasis showing cellular monomorphism, moderate pleomorphism with occasional intranuclear inclusion.

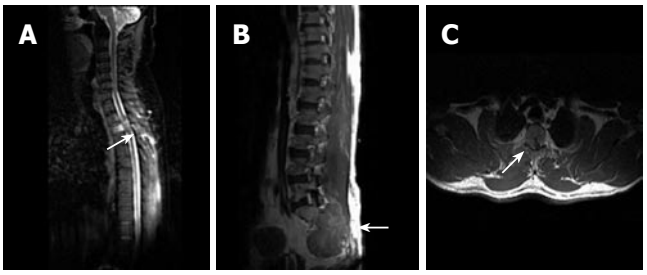

Figure 2 **A**: Sagittal MR image of cervicodorsal spine showing vertebral lesions with most prominent D3 vertebral lesion (arrow); **B**: Post contrast sagittal T1W SE MR image of the same patient showing lesion involving S2, S3 vertebrae (arrow) with associated large soft tissue component; **C**: T1W SE axial post contrast MR image showing right lateral epidural soft issue enhancing component (arrow) causing cord compression.

poorly differentiated neoplasm. Serum alpha feto protein (AFP) level was more than 500 IU/mL (normal AFP level < 10 IU/mL). Ultrasound if testis, upper GI endoscopy, CT chest scan were unremarkable but CT scan of the abdomen revealed a large well defined hypodense lesion, 7.5 cm × 5.8 cm in dimensions with areas of necrosis, in the right liver lobe, in segment V, with no involvement of the portal vein. Additionally there was a destructive lesion in the right ala of the sacrum associated with a bulging soft tissue component, 5.3 cm × 3.9 cm in dimensions, which corroborated with MRI spine and bone scan findings. The patient underwent CT guided FNAC and Tru-cut biopsy of the right liver lobe lesion. Histopathological examination (HPE) was suggestive of poorly differentiated primary HCC, showing increased trabecular thickness and cellular pleomorphism with vesicular nuclei (Figure 1). Viral marker profile was positive for Hepatitis B surface antigen (HbsAg) and was non-reactive to hepatitis C virus. He was diagnosed as having primary HCC with bone metastases. The option of palliative chemotherapy was declined by the patient and he was started on palliative external beam radiotherapy (EBRT) to the right pelvis in view of pain. He developed acute onset paraplegia along with urinary retention shortly after initiation of EBRT. Immediate intravenous Solumedrol and other supportive measures were taken. Whole spine MRI was done and revealed multiple vertebral metastases at D2-D3, L1, L5, S1,S3 vertebral levels (Figure 2A and 2B) and epidural deposits with extradural cord compression at D2 and D3 vertebrae (Figure 2C). FNAC from vertebral metastases (Figure 3) revealed metastatic carcinoma consistent with primary HCC. Decompression and fixation surgery was suggested, but the family did not opt for any surgical intervention. He continued to receive EBRT for SCC. Although, there was symptomatic improvement in the pain intensity, there was no significant improvement in the neurological status. After further discussion with the family, oral chemotherapy was performed with Capecitabine and Thalidomide with no response after two cycles. Despite prompt treatment of SCC with steroids and EBRT, the patient succumbed to the disease five months after primary diagnosis.

Case 2

A 52-year-old non-alcoholic man presented with complaints of heaviness and discomfort in the right upper abdomen of two months duration. Whole abdomen ultrasound done elsewhere was suggestive of liver mass. Clinical examination revealed presence of mild hepatomegaly. Laboratory investigations showed a normal hematological profile and mild derangement of liver function tests (LFTs). Viral marker profile was positive for HbsAg and non-reactive to HCV. Whole abdomen CT scan revealed mild hepatic enlargement with an ill-defined hypodense lesion of 3.8 cm in diameter, in segment V and VI of the liver with no involvement of portal vein. His AFP levels were 3540 IU/mL. Magnetic resonance cholangio pancreatography (MRCP) revealed a nodular outline and texture of liver consistent with cirrhosis, with a focal lesion in segment V and VI, measuring 3.5 cm × 4.0 cm × 4.1 cm. He underwent CT guided FNAC from right liver lobe mass which was suggestive of primary HCC. Whole body 99 m Tc bone scan revealed no evidence of bone metastases. He was diagnosed as having primary HCC with no evidence of extra-hepatic spread. He underwent hepatic segmentectomy. HPE of the resected specimen of liver segment revealed poorly differentiated HCC with cirrhosis and regenerative nodules. CT scan of the abdomen after surgery as a part of revaluation revealed a new focal hypodense lesion measuring 2.4 cm × 2.5 cm, in the caudate lobe of the liver and another

ill defined hypodense lesion in the right lobe of the liver with an expansile lesion with soft tissue component in the right seventh rib. Whole body 99 mTc bone scan showed irregular radiotracer uptake in the right seventh rib anterolaterally. He underwent FNAC from right seventh rib, which revealed metastatic deposit of HCC. There was a rise in serum AFP level from 3540 IU/mL at the time of diagnosis to 7920 IU/mL, suggestive of relapse. He received palliative chemotherapy with Gemcitabine and Cisplatin. CT scans after three cycles of chemotherapy revealed complete regression of caudate lobe, right liver lobe and right seventh rib lesion with a new development of a well defined lytic lesion in L2 vertebra. He also had pain in the right hip joint. Chemotherapy was continued with the same protocol for three more cycles. Subsequent CT chest scan revealed development of a new lesion in the left third rib with destruction of the left transverse process of D3 vertebra along with erosion of D5 vertebra. CT guided FNAC from the third left rib lesion was positive for malignant cells, consistent with primary in liver. He received palliative EBRT to the symptomatic areas like right hip joint, C7 to D7 vertebrae and L1 to L4 Vertebrae. One month later, he developed paraplegia with complete loss of bladder and bowel control. MRI of Dorso-lumber spine was suggestive of metastatic lesion at dorsal and lumbo-sacral spine with cord compression at the D6 vertebral level. The patient underwent D5 to D6 laminectomy with posterior fixation. HPE of laminectomied specimen was reported as metastatic carcinoma consistent with primary HCC. Postoperatively there was no significant improvement in the paraplegia and he did not regain bladder and bowel control. He was supported with intravenous steroids, bisphosphonates and physiotherapy. After laminectomy, although palliative EBRT was planned to the dorsal spine, (D4 to D7) vertebrae and to the lower dorsal spine, his general condition deteriorated and he succumbed to his illness 11 mo after the primary diagnosis.

Case 3

A 70-year-old man who was alcoholic, diabetic and a smoker, presented with complaints of pain in the right side of the chest. Clinical examination revealed mild hepatomegaly and pallor. Whole abdomen CT scan revealed left liver lobe mass suggestive of primary HCC with portal vein thrombosis and abdominal lymphadenopathy. He underwent MRCP and MR angiography, which revealed a soft tissue lesion in the left lobe of the liver with portal vein thrombosis and abdominal lymphadenopathy, with no signs of cirrhosis of liver. His hematological and biochemical profile were within normal range. HbsAg and HCV were not reactive in viral marker profile. His serum AFP level was 250 IU/mL. Tru-cut biopsy of left liver lobe lesion was consistent with the diagnosis of HCC. Whole body 99 mTc bone scan revealed no evidence of bone metastases at the time of diagnosis. Chemotherapy was given with Gemcitabine and Capecitabine. After the second cycle, he had sudden onset of weakness of bilateral lower limbs. Neurological examination revealed paraplegia with sensory loss below D5 level with moderate sphincteric involvement. Whole spine MRI showed extradural cord compression at D4 and D5. Whole body 99 mTc bone scan revealed abnormal areas of radiotracer uptake in D4 and D5 vertebrae, consistent with bone metastases. Emergency D1 to D4 vertebral laminectomy was performed with posterior fixation from C7 to D5 vertebrae. HPE of laminectomied specimen showed metastasis from HCC. He received EBRT to the spine along with aggressive physiotherapy and with other supportive measures after surgery. He achieved good neurological and symptomatic recovery, and was discharged in the hemodynamically stable condition. As the patient was not willing to receive further treatment, he was followed up on palliative supportive care. However, he succumbed to the disease four months after the diagnosis.

Case 4

A 62-year-old normotensive, non diabetic, nonalcoholic man presented with complaints of pain in neck and lower back of one month duration. Pain radiated from neck to fingers bilaterally. He also had difficulty in walking due to radicular pain. Clinical examination revealed painful neck movement and mild hepatomegaly. Hematological profile was normal mild derangement of liver enzymes. Both HbsAg and HCV were non-reactive. Whole abdomen ultrasound revealed a well defined solid mass, measuring 8.4 cm × 4.9 cm, in the right lobe of the liver, with heterogeneous echotexture. Upper abdomen CT scan also revealed enlarged liver with a large hypodense lesion, measuring 10.5 cm × 10.1 cm, in the right lobe of the liver. Whole spine MRI was suggestive of multiple bone metastases in C5-C6, D9, D11-D12 and L4 vertebrae and the bodies of D9 and L4 vertebrae revealed wedge compression fracture. A small anterior epidural space soft tissue collection was also present, causing compression of the thecal sac at L4 vertebral level. Whole body 99 mTc bone scan revealed increased radiotracer uptake in D7, D9, D11-D12 and the L4 vertebrae, sacrum and bilateral sacroiliac joints, suggestive of bone metastases. He underwent CT guided FNAC from right liver lobe mass which revealed primary HCC. FNAC from vertebra was consistent with metastasis from primary HCC. Serum AFP level was elevated to 121 IU/mL at the time of diagnosis. He was diagnosed as having primary HCC with multiple bone metastases with SCC, manifesting primarily as radiculopathy. The patient and the family were given the options of surgical decompression as well as chemotherapeutic management, which they were not inclined for in view of disseminated disease. Hence, he was started on palliative EBRT to L2-L5 vertebrae and to C4-C7 vertebrae. EBRT to the thoracic spine was performed later. After completion of radiotherapy (RT) there was symptomatic improvement in the pain intensity. However, after EBRT, there was progression of SCC leading to development of paraparesis with urine incontinence. He was managed conservatively in view of deteriorating general condition. He died from his illness three months after the primary diagnosis.

DISCUSSION

HCC is the most common solid organ tumor with a high mortality[2]. Most patients with HCC present with right upper quadrant pain or an abdominal mass due to the

presence of hepatomegaly[7,8,9]. Rarely though, the patients may have initial symptoms related exclusively to the extra-hepatic metastases[6]. Serum AFP level is the most useful serum tumor marker for primary HCC. Serum AFP level is elevated in most of the patients, which is highly specific for a tumor larger than three cm in diameter, and also is of prognostic value as rise after initial effective chemotherapy or surgery suggests a relapse[3]. This was evident from the fact that AFP level was elevated in all our cases and relapse occurred after hepatic segmentectomy in the second patient corresponded to the elevation of AFP levels.

Extra-hepatic spread from HCC is not uncommon and reported to be 30%-78% at autopsy examination. Bone metastases are rare with an incidence of only about 2%-20%[2-5,10,11]. Although incidence of metastasis of HCC to the bones is low, recent reports have shown an increasing incidence and is estimated to be about 28%[1].

The most frequent sites of the bone metastases are ribs, spine, femur, pelvis and humerus according to Khulman *et al*[2]. Patients with bone metastases most often present with pain as the principal symptom[3,7]. Two of our patients presented with the chief complaints of backache. Very rarely patient may present with bone pains without any symptom of underlying hepatic pathology, as seen in our first case.

In the majority of cases, vertebral body metastases result from hematogenous dissemination of tumor which is evident by the vertebral column bone marrow involvement[6,10]. Radiologically, bone metastases from HCC appear osteolytic on plain films. All the four patients in our series had osteolytic lesions. They are destructive, expansive and often associated with large soft tissue masses[2-6,10]. Conventional radiography is however, not a very sensitive modality for the diagnosis of early bone metastases as the cancellous part of the bone is usually the first site of bone metastases and cortical part of the bone is responsible for most of the bone density depicted on plain X-Ray films[6]. For the same reason the bone scan is also less sensitive. MRI is most helpful for early diagnosis as well as delineation of the extent of metastases[2,6].

The histological appearance of the bone metastases from primary HCC is similar to that of the primary tumor, with positive bile staining[2,4]. Recent insight into the causation of bone metastases of HCC has been correlated with angiogenesis[8]. Significant hemorrhage from metastatic lesions is reported to occur either spontaneously or after biopsy of the lesion[2].

The level of vascular endothelial growth factor (VEGF) has been reported to be elevated in HCC with bone metastases[8]. VEGF, the most important angiogenic factor, has been shown to stimulate bone resorption through its effects on osteoclasts[8,12]. Thus in the era of targeted therapy, VEGF could be an important target for the treatment of these tumors. In their study on metastatic HCC, S. Kummar *et al* have evaluated TNP-470, a derivative of fumagillin and a potent angiogenetic inhibitor, as a treatment for experimentally induced HCC in animal models[8]. Similarly, the serum levels of C- terminal telopeptide of type 1 collagen are also significantly elevated in patients with bone metastases[13].

The overall frequency of the malignant SCC has been reported to be approximately 5% in cancer patients. In approximately 95% of cases, SCC is caused by extradural metastases from tumors involving the vertebral column. Thoracic spine (70%) is most commonly involved as compared to the lumbosacral (20%) and the cervical spine (10%)[12,14]. Three cases reported by us had SCC at thoracic vertebrae level and all of them had extradural compression. Although SCC is rare with HCC, the symptoms and involvement are similar to other primary tumors, commonly leading to SCC, such as lung, breast and prostate cancer.

Omura *et al* in 1989 described a case of a 57-year-old male with primary HCC diagnosed when he developed paraplegia secondary to a SCC due to vertebral tumor. After laminectomy, the tumor histology was reported to be metastatic HCC[15]. Kantharia *et al* in 1993, also described a case of radiculopathy and rapidly developing SCC, due to bone metastases and diagnosed as HCC at autopsy[6]. Pinazo Seron *et al* in 1999 reported a case of a 55-year-old man, who had alcoholic cirrhosis and HCC. This patient developed SCC due to soft tissue epidural metastases, seated at the paravertebral zone. Plain radiography and bone scan were normal and diagnosis was achieved by MRI and FNAC[16]. Cho *et al* in 2002 reported a case of pathologically confirmed HCC who developed lower leg weakness, which was found to be due to spinal metastases as evidenced by MRI spine. This patient received emergency radiotherapy (RT) and recovered from SCC[17]. Melicher *et al* in 2002 also described a case of asymptomatic liver mass of uncertain histology of one year duration. He presented with back pain and developed signs of SCC, and he underwent laminectomy, which established the diagnosis of metastatic HCC[18].

Cord compression occurs due to invasion of epidural space, most often as a direct extension of vertebral body metastases. There are various routes of the epidural invasion by tumor cells, hematogenous being the most common mode of spread. Hematogenous spread occurs directly or *via* the involvement of Batsons venous plexuses[19].

In metastatic SCC, back pain is not only the most common symptom but also the earliest manifestation of SCC, as seen in two of our patients. First sign after development of pain is weakness due to myelopathy. Once lost, neurological functions cannot be regained in most of the patients[12,20,21].

Though various modalities such as plain radiography, CT scan, CT-myelography and MRI can be used to evaluate SCC, whole spine MRI is the best method of evaluating epidural SCC. Apart from being most sensitive, cost-effective and noninvasive, MRI is also helpful in distinguishing between benign and malignant causes of SCC[20,21]. In all our patients, SCC was diagnosed with the help of MRI spine, which revealed the site of extradural cord compression with precision. Presently CT-myelography is used only for patients in whom MRI is contraindicated[21].

The pre-treatment degree of neurologic dysfunction is the strongest predictor of therapeutic outcome. However, the most important weapon against the prevention of devastating complications of SCC is the high index

of suspicion and the awareness that SCC is a potential oncologic emergency[20]. Thus the development of any new pain or any change in the character of the pain mandates complete neurological examination along with MRI whole spine as a screening modality to rule out early signs of SCC[21].

The treatment modalities available for SCC are individualized with a definite role of corticosteriods, RT, chemotherapy and surgery. Corticosteriods act by relieving edema and help preserve neurological function. They may also improve overall outcome after specific therapy[19,22,23].

RT is an important part of the management of SCC and it helps in pain relief, cytoreduction of tumor, prevention of progressive neurologic dysfunction and structural damage to the cord. RT reduces pain in approximately 70%, improves motor function in 45% to 60%, and reverses paraplegia in 11% to 21% of the patients. The outcome of RT is related to the neurological status prior to the treatment and the radiosensitivity of the tumor. RT should also be given following surgery in patients who have not previously received radiation[24-27]. Our third patient was given EBRT to the affected region after emergency posterior laminectomy was performed.

Surgical interventions are usually indicated in situations where the diagnosis is unknown, in cases of spinal instability or compression by the bone requiring prompt relief of pressure related symptoms[12,20,27]. These four cases suggest that surgery should be the integral part of SCC management in HCC. Our first patient immediately received RT after developing SCC, with no improvement in neurological status, and in the second patient, SCC developed at the vertebra which was earlier irradiated. RT did not help prevent paraparesis in the fourth patient. Whereas, the third patient who underwent emergency laminectomy followed by EBRT had best palliation. Surgery should also be considered in the patients who fail to respond to RT or deteriorate further while on RT and the patients who have received maximal allowable radiation dose to the spinal cord[12,20]. The surgical approach should be determined based on the location of vertebral involvement and the direction of compression[20]. The two main surgical approaches used for decompression are laminectomy with posterior fixation and anterior decompression of the spine with reconstruction. Anterior decompression of the spine with mechanical stabilization has been accepted as the surgical intervention of choice for anterior vertebral body involvement[21,28]. Our second and third patient underwent posterior laminectomy for emergency decompression without any immediate morbidities. But the aim is to diagnose SCC in the early stage so that emergency decompressive surgery will not be needed.

CONCLUSION

Extra-hepatic spread of HCC is usually to the lungs and regional lymph nodes, but rarely to the bones. SCC due to vertebral bone metastases is a rarer phenomenon. SCC is an oncologic emergency and treatment delay can lead to irreversible consequences. These four cases highlight unusual metastatic presentation of HCC, in the form of bone metastases resulting in SCC. High index of suspicion is required to achieve good outcome in patients who are at risk to develop SCC secondary to bone metastases. Thus, any change in the character of already existing bone pain or new development of pain mandates a complete neurological examination along with MRI of the whole spine to rule out cord compression. Although, radiation, steroids and surgery can be used as treatment modalities, surgery followed by EBRT can result in better palliation. But the aim is to diagnose SCC at an early stage so as to prevent the devastating consequences of this complication.

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CASE REPORT

Fulminant hepatic failure caused by *Salmonella paratyphi A* infection

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Abstract

We report a case of fulminant hepatic failure associated with *Salmonella paratyphi A* infection, in a 29-year-old patient who was admitted to the intensive care unit (ICU) with fever of two days, headache and vomiting followed by behavioural changes and disorientation. On examination, the patient appeared acutely ill, agitated, confused, and deeply jaundiced. Temperature 38.5℃, pulse 92/min, blood pressure 130/89 mmHg. Both samples of blood grew *S. paratyphi A*, which was sensitive to ceftriaxone and ciprofloxacin. Ceftriaxon was administered with high-dose dexamethasone. Two weeks after treatment with ceftriaxon, the patient was discharged in satisfactory condition.

Key words: Typhoid hepatitis; Fulminant hepatic failure; *Salmonella paratyphi A*

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INTRODUCTION

Fulminant hepatic failure is a dramatic clinical syndrome characterized by massive necrosis of liver cells[1]. It is caused by acute viral hepatitis A, B, C, D, E (40%), other viruses, drugs, Weil's disease, Wilson's disease, acute fatty liver in pregnancy, ischemic hepatitis, acute Budd-Chiari, malignant infiltration or bacterial infection including *salmonella*.

Although salmonella hepatitis is a rare presentation of typhoid fever, fulminant hepatic failure is extremely uncommon, few cases were reported with such presentation. To our knowledge, this is the first reported case of fulminant hepatic failure in the state of Qatar, associated with *Salmonella paratyphi A* infection.

CASE REPORT

A 29-year-old Nepali male patient admitted to the intensive care unit (ICU) through accident and emergency (A&E) department with fever for two days, headache and vomiting followed by behavioral changes and disorientation. The patient returned back from his country two months ago. No other history of possible relevance was found. On examination, the patient appeared acutely ill, agitated, confused, and deeply jaundiced. Temperature was 38.5℃, pulse 92/min, and blood pressure 130/89 mmHg. Examination of the nervous system showed a disoriented patient, moving all his limbs, with incoherent speech and negative meningeal signs. Examination of the abdomen revealed mild splenomegaly. Chest and heart were unremarkable. Hemoglobin level was 150 g/L, total leucocyte count 5000/μL (60% neutrophils, 31% lymphocytes) and adequate number of platelets. Blood chemistry showed asparate aminotransferase (AST) level of 966 IU/L, alanine amino-transferase (ALT) 176 IU/L, alkaline phosphatase 267 IU/L, total bilirubin 180 μmol/L, direct bilirubin 109 μmol/L, total proteins 74 g/L, albumin 38 g/L, urea nitrogen 3 mmol/L, creatinine 44 μmol/L, sodium 135 mEq/L and potassium 3.6 mEq/L, bicarbonate 22 mmol/L, Ca 2.2 mmol/L , ammonia level 100 μmol/L (normal up to 50 μmol/L), prothrombin time 30.8 s and an international normalized ratio (INR) of 3.1. Macroscopic and microscopic examination of urine and stool were normal. Viral markers for hepatitis A, B, C, Monospot test and cytomegaloirus serology were negative. Antismooth muscle antibody and antimithocondrial antibody were negative. Peripheral smear for malaria was negative on several occasions. Lumber puncture was done, and CSF study was normal.

Computerized tomography (CT) scan of the head with intravenous contrast was normal. Electroencephalography showed findings suggestive of metabolic encephalopathy. Chest X ray was normal. Ultrasonography of abdomen revealed enlarged spleen. Rest of abdomen including liver, gall bladder, and kidney were all within normal limits.

A CT scan of the abdomen, with intravenous contrast showed moderate spleen enlargement and liver enlargement with homogeneous parenchyma. There was no evidence of abscess or tumor. No evidence of mass or fluid collection was seen in the abdomen or pelvis. Kidneys and

Table 1 Laboratory data on admission and discharge

	Alanine amino-transferase (ALT)	Asparate aminotrans-ferase (AST)	Alkaline phosphatase (ALP)	Total bilirubin
On admission day	176 IU/L	966 IU/L	267 IU/L	180 μmol/L
On discharge day	65 IU/L	105 IU/L	110 IU/L	33 μmol/L

pancreas were within normal limits. Echocardiography was normal. Magnetic resonance image (MRI) of the abdomen with MR cholangiography showed peri-portal edema within the liver and collapsed gall bladder, suggesting the possibility of hepatitis.

The patient was admitted to the ICU as a case of fulminant hepatic failure evidenced by marked elevation of hepatocellular enzymes, prolonged prothrombin time, hyperbilirubinemia and hyperammonaemia. The patient was given vitamin k and fresh frozen plasma to correct the prolonged INR, and Lactulose (enema and oral). Broad-spectrum antibiotic and high dose dexamethasone (3 mg/kg loading dose over 30 min, followed by 1 mg/kg every six hours for two days) were initiated. Cultures of urine were sterile and no pathogen was isolated from stool culture.

However, both samples of blood grew *S. paratyphi A*, which was sensitive to ciprofloxacin and ceftriaxone. Once the culture report was available the treatment was changed to ceftriaxone 2 gm twice daily. After one week of therapy, the patient was afebrile and oriented, and the dose of ceftriaxon was reduced to 2 gm daily for one more week. Two weeks after treatment with ceftriaxon, the liver enzymes dropped obviously (Table 1). Consequently, the patient was discharged in satisfactory condition.

DISCUSSION

Typhoid fever may be an important cause of illness and death for centuries, although historical accounts do not clearly distinguish it from other febrile illnesses. It has been implicated in the death of Alexander the Great, in 323 B.C.[2].

The causative organism widely spreads in all parts of the world, although the disease is more prevalent in developing countries than in developed ones. The most common cause of typhoid fever is *Salmonella enterica* serotype typhi, although salmonella of other serotypes, particularly *Salmonella enterica* serotype paratyphi A, can cause a similar enteric fever. *Salmonella enterica* serotype typhi has no animal reservoir.

In typhoid, involvement of liver is a consistent feature[3,4]. Typhoid hepatitis is a rare presentation of typhoid fever, clinically suspected in patients with persistent fever , hepatomegaly and jaundice and especially in cases whose liver function tests show predominantly conjugated hyperbilirubinemia, modest elevation of liver enzymes and negative serology for viral hepatitis[5,6].

The differential diagnosis of fever in the icteric phase of hepatitis, include: viral hepatitis, malaria hepatitis, Weil' s disease, typhoid hepatitis, autoimmune hepatitis and drug induced hepatitis.

In this patient, viral markers for hepatitis A, B, C, Monospot test and cytomegalovirus serology were negative. Peripheral smear for malaria was negative on several occasions. Antismooth muscle antibodeis (Ab) and antimithocondrial Ab were negative and whether the patient had taken any hepatotoxic drugs was unknown.

The common complications of typhoid fever include relapse, perforation and hemorrhage from bowel ulcerations[3]. Extreme hepatic dysfunction with hepatic encephalopathy is a rare coexisting complication in salmonella hepatitis.

The pathogenesis of severe hepatic involvement in salmonella infection may be multifactorial, involving endotoxin, local inflammatory and/or host immune reactions.

In typhoid hepatitis, liver biopsy would have shown pathognomonic lobular aggregates of Kupffer's cells-so-called typhoid nodules, but liver biopsy was not performed in this case[2].

Although, viral markers for hepatitis E and leptospiral test were not made in this case, the diagnosis of fulminant hepatic failure due to *S. paratyphi* was based mainly on three facts: first, positive blood culture for *S. paratyphi A*; second, elevated alkaline phosphatase level, aspartate aminotransferase (AST) level higher than alanine aminotransferase (ALT) , and only mild prolongation of the prothrombin time[7]; and third, excellent response to ceftriaxon and dexamethasone[7].

The most important aspect of treatment for typhoid fulminant hepatic failure is to provide good intensive care support, recognize the condition promptly and initiate early dexamethasone and proper antibiotics. Monitoring the complications and instituting appropriate therapy are also critical.

The current drug of choice for adults is ciprofloxacin, which combines little documented resistance with excellent penetration into macrophages and the biliary system. This may lower the rate of relapse and chronic carrier states. Alternative antibiotics can be used if sensitivities are known or suspected resistance is low[8]. The current drug of choice for children and pregnant women is parenteral ceftriaxone[9].

Thus, in patients from endemic areas, typhoid hepatitis should be considered in the differential diagnosis of fulminant hepatic failure since early institution of specific therapy in these cases yields a good prognosis.

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CASE REPORT

Multi-limb necrotizing fasciitis in a patient with rectal cancer

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Abstract

Necrotizing fasciitis is a devastating soft tissue infection affecting fascias and subcutaneous soft tissues. Literature reviews have identified several related risk factors, including malignancy, alcoholism, malnutrition, diabetes, male gender and old age. There are only scanty case reports in the literature describing its rare association with colorectal malignancy. All published cases are attributed to bowel perforation resulting in necrotizing fasciitis over the perineal region. Isolated upper or lower limb diseases are rarely identified. Simultaneous upper and lower limb infection in colorectal cancer patients has never been described in the literature. We report an unusual case of multi-limb necrotizing fasciitis in a patient with underlying non-perforated rectal carcinoma.

Key words: Necrotizing fasciitis; Soft tissue infection; Colorectal cancer; Malignancy; Group G streptococcus

Liu SYW, Ng SSM, Lee JFY. Multi-limb necrotizing fasciitis in a patient with rectal cancer. *World J Gastroenterol* 2006; 12(32): 5256-5258

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INTRODUCTION

Necrotizing fasciitis is a devastating soft tissue infection affecting fascias and subcutaneous soft tissues[1]. Two distinctive clinical entities are recognized: hyperacute and subacute variants[2]. Hyperacute necrotizing fasciitis usually manifests as a rapidly progressing infection with resultant multi-organ failure due to group A streptococcus or clostridium species[3]. Contrarily, polymicrobial infection is implicated in subacute necrotizing fasciitis that represents a slowly evolving disease with less fulminant systemic complications[4]. Regarding its etiology, necrotizing soft tissue infection can be secondary to trauma, wound infections, perianal or urogenital abscesses, decubitus ulcers or idiopathic in origin[5]. Malignancy, alcoholism, malnutrition, diabetes, male gender and old age are general risk factors associated with necrotizing fasciitis[6,7]. There are only scanty case reports in the literature describing its rare association with colorectal malignancy[8-13]. All these cases are attributed to bowel perforation resulting in necrotizing fasciitis over the perineal or abdominal region. Isolated upper or lower limb diseases are exceedingly uncommon. Multi-limb necrotizing fasciitis is even rarer. We report here the first case of multi-limb involvement of necrotizing fasciitis in a patient with underlying rectal carcinoma.

CASE REPORT

A 56-year-old male patient, who had a medical history of alcoholic cirrhosis, poorly-controlled diabetes mellitus and mild renal impairment, presented with an one-month history of per rectal bleeding. Digital rectal examination revealed a non-obstructing rectal tumour situated at 8 cm above the anal verge. Tumour biopsy was taken through proctoscope and the histology confirmed it to be an adenocarcinoma. No abdominal discomfort or increase in per rectal bleeding was reported after the procedure. However, one day after the tumour biopsy, he complained of bilateral lower limb pain over the pre-existing ankle edema. He denied any previous history of trauma. No wound or skin abrasions could be identified over both lower limbs. In addition to the swollen lower limbs, he was noticed to have unexplained sinus tachycardia (pulse rate 100 per min) despite normal haemodynamic status and oxygen saturation. Examination of other organ systems was unremarkable. The arterial blood gas, white cell counts, chest radiograph and electrocardiogram were normal. Though Doppler ultrasound did not show any evidence of deep vein thrombosis in both lower limbs, low molecular weight heparin was commenced as pulmonary embolism was not excluded.

His clinical condition rapidly deteriorated over the next 24 h when he developed acute on chronic renal impairment, hypotension and worsening metabolic acidosis that necessitated inotropes and hemodialysis support in the intensive care unit. Urgent ultrasound of the abdomen did not reveal any evidence of obstructive uropathy. He became hypothermic (core temperature 32℃), drowsy and mentally confused. Blood parameters revealed marked leukocytosis and disseminated intravascular coagulation. Empirical intravenous antibiotics including augmentin (amoxycillin and clavulanate), clindamycin and metornidazole were commenced in view of the clinical

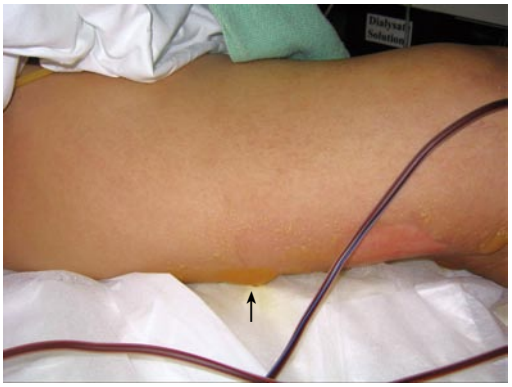

Figure 1 Enlarging blisters over the thigh. One of the blisters is indicated by black arrow.

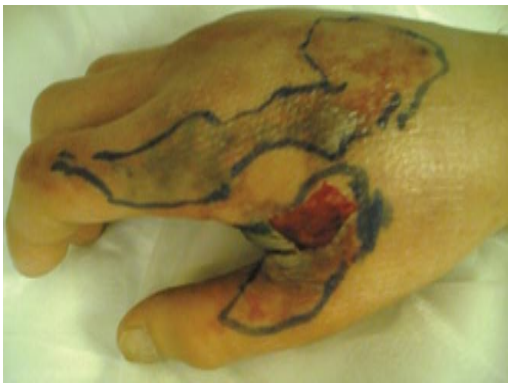

Figure 2 Necrotic skin patches over dorsum of hand.

Table 1 Literature review of necrotizing fasciitis associated with colorectal malignancy

Authors	Yr published	Patient sex	Colorectal cancer type	Predisposing factors	Location of necrotizing fasciitis	Bacteria cultured	Outcome
Dewire *et al*[8]	1992	Male	Sigmoid	Bowel perforation	Fournier's gangrene	*E coli*, Bacteroides fragilis, enterococci, anaerobes	Alive
Lam *et al*[9]	1996	Male	Sigmoid	Bowel perforation	Psoas abscess to upper thigh	*E coli*, Bacteroides fragilis, Edwardsiella tarda, staphylococcus, & Group G streptococci	Death
Gould *et al*[10]	1997	Male	Sigmoid	Bowel perforation	Fournier's gangrene	Group F streptococci, anaerobes	Death
Lawrentschuk *et al*[11]	2003	Male	Rectum	Perforation with abscess	Fournier's gangrene	*E coli*, Enterococcus, anaerobes & staphylococcus epidermidis	Alive
Gamagami *et al*[12]	1998	Male	Rectum	Perforation with abscess, Diabetes	Fournier's gangrene	*E coli*, enterococci	Alive
Ku *et al*[13]	2006	Female	Transverse	Perforation with invasion to rectus fascia	Abdominal wall	Klebsiella pneumonia	Alive

features of sepsis. Shortly after his stay in the intensive care unit, blisters (Figure 1) were discovered on both swollen lower limbs. Necrotizing fasciitis was suspected and ultimately confirmed with surgical exploration 12 h after the appearance of blisters. Initially, fasciotomy and excisional debridement were endeavored in an attempt to preserve the lower limbs. Meanwhile, high dose penicillin was introduced to replace augmentin. Nevertheless, the worsening clinical sepsis and migratory soft tissue necrosis inevitably resulted in bilateral above knee amputation. Microbiological cultures of blood and necrotic tissues invariably grew group G streptococcus only. Histological examination of the excised tissue also confirmed the diagnosis of necrotizing fasciitis. One day afterwards, similar features of skin changes developed over both upper limbs (Figure 2) that required repeated excisional debridements. In spite of limb amputation, surgical debridement and parenteral antibiotics, he developed fulminant group G streptococcal septicaemia and deteriorated rapidly until he succumbed to multi-organ failure six days after the onset of sepsis. Throughout his disease course, the perineum, groin and abdominal wall remained uninvolved.

DISCUSSION

In the English literature, there are only six reported cases of necrotizing fasciitis associated with colorectal malignancy (Table 1). In these cases, necrotizing fasciitis was all preceded by perforation of colorectal tumours. All except two cases[8,10-12] presented as Fournier's gangrene. One case manifested as psoas abscess with spreading infection to the thigh after retroperitoneal perforation of a sigmoid tumour[9]. The other one involved the anterior abdominal wall as a result of direct tumour invasion and perforation from the transverse colon[13]. In essence, perforated colorectal tumours represent an entry focus for bacterial translocation that might penetrate the subcutaneous soft tissues, leading to necrotizing infection[14]. To our knowledge, our patient is the first reported case of disseminated necrotizing fasciitis affecting all limbs in a non-perforated rectal tumour.

With regard to its etiology, we postulate three causes for such a fulminating disease in our patient. First, chronic subcutaneous tissue edema due to cirrhotic hypoalbuminaemia predispose him to subcutaneous tissue infection[6]. In addition, systemic immunosuppression could well be secondary to the cumulative effects of malignancy, diabetes mellitus and cirrhosis[7]. Lastly, transient bacteraemia might have arisen as a consequence of bacterial translocation emanated from occult necrosis of his rectal tumor[14]. Interestingly, there was a close temporal association between tumour biopsy and the onset of necrotizing sepsis in our patient. Transient bacteraemia following endoscopic tissue biopsy has been reported[15]. However, Gram negative organisms were the main bacteria retrieved and it was elusive to verify the causative association between rectal biopsy and necrotizing soft tissue infection in our patient.

So far as the bacteriological origin was concerned, group G streptococcus was the only culprit in our patient. In 1935, Lancefield, an American microbiologist, first

described a Gram positive facultative anaerobic coccus as group G streptococcus that constitutes parts of the normal flora of human skin, respiratory tract and gastrointestinal tract[16-18]. Although it has been rarely implicated in necrotizing fasciitis[19], its associated mortality could be comparable to those induced by streptococcus pyogenes[20], suggesting that group G streptococcus, being a normal flora of the gastrointestinal tract, could represent a potentially life-threatening pathogen for all patients[20].

Regardless of the disparity in pathological and microbiological etiologies, the mortality rate of necrotizing fasciitis remains high. High index of suspicion and prompt surgical intervention are the cornerstone of treatment for improving the disease outcome[7]. We believe that delay in diagnosis and surgical treatment contributes to the demise of our patient.

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Cardiac mucosa indicates risk for Barrett esophagus

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TO THE EDITOR

With interest we read the article by Bani-Hani *et al*[1] entitled "Pathogenesis of columnar-lined esophagus", which has been published in the recent issue of *World Journal of Gastroenterology*. The review profoundly adds to our understanding of columnar-lined esophagus (CLE) and clearly indicates that CLE represents an acquired condition and develops as a consequence of gastroesophageal reflux disease (GERD)[1]. However, it should be pointed out that inclusion of CLE-histopathology helps to define those at risk for dysplastic and malignant transformation. Histopathology characterizes *nondysplastic* and *dysplastic* (low-, high grade dysplasia) CLE[2-4]. Going in line with the *Paull-Chandrasoma* classification[2], *nondysplastic* CLE includes oxyntocardiac mucosa, cardiac mucosa, multilayered epithelium (a mixture of basal layer of squamous epithelium and cardiac mucosa) and cardiac mucosa with goblet cells (i.e. intestinal metaplasia; *Barrett* esophagus, BE). In contrast to cardiac mucosa, goblet cells have never been detected within oxyntocardiac mucosa[2,3]. Consequently, following a 4-quadrant biopsy protocol of the esophagogastric junction (including squamous and gastric type mucosa, irrespective of the presence or absence of endoscopic CLE) presence of oxyntocardiac mucosa in all biopsies indicates absence of risk to progress towards intestinal metaplasia and dysplasia[2,3]. In contrast to that, presence of cardiac mucosa indicates risk to undergo intestinal metaplasia and progress towards dysplasia and adenocarcinoma of the esophagus (annual incidence for BE 0.2%-2.0%). Controversy still exists if CLE is esophageal or gastric. Normally the esophagus is lined by squamous epithelium and the stomach is covered by oxyntic mucosa. Recently Chandrasoma *et al*[4] showed that CLE, but not oxyntic mucosa, is present above submucosal glands and adjacent to submucosal gland ducts. Since submucosal glands are absent in the stomach, CLE is considered to be esophageal, irrespective of endoscopic appearance[2-4]. Taken together, CLE represents the morphologic consequence of GERD. GERD should be included into histopathologic routine. Hopefully, future studies will evaluate the incidence and prevalence of CLE subtypes in the normal population for identification of those at risk for intestinal metaplasia and adenocarcinoma of the esophagus.

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Meetings

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Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

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26-29 November 2006
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Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
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26-30 April 2006
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Prague hepatology 2006
14-16 September 2006
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Both personal authors and an organization as author

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

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Issue with no volume

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Books

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

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